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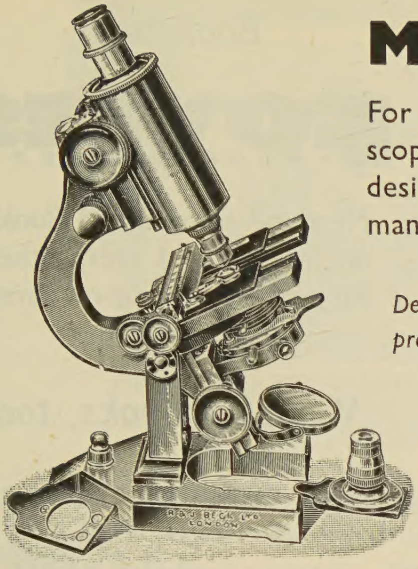

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The Activities of the Tube Feet of *Asterias rubens* L.

I. The Mechanics of Movement and of Posture

BY

J. E. SMITH

(*Zoological Department, University of Cambridge*)

With seven Text-figures

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I. INTRODUCTION

ASTERIAS RUBENS, under the normal circumstances of its life in tide pools and in the deeper waters of the continental shelf, displays a range of activities which include locomotion, the capture of food, and the maintenance of a posture in which the oral surface is kept in apposition to the substratum. These and other organismal activities require an harmonious integration of movement of the different parts and organs of the body, chief among which are the tube feet and the muscular walls of the arms. These integrated movements of widely separated systems contrast vividly with the strictly autonomous behaviour of the spines and pedicellariae situated on various parts of the lateral and dorsal surfaces of the arms and disk.

It is apparent from the work of Romanes and Ewart (1881), Preyer (1886), Demoor and Chapeaux (1891), Jennings (1907), Mangold (1908), and Diebschlag (1938), among other authorities, that the ability of the tube feet and myodermal wall of the arms to take part in organismal responses is dependent on an innervation which includes nervous connexion with the neurones and tracts of the radial nerve cord and circumoral nerve ring, and that the autonomous behaviour of the spines and pedicellariae is a consequence of their being innervated solely through the peripheral plexus of the dorsal sheath (i.e. the integument lateral to the tube feet). Some of the implications of peripheral and central innervation in terms of the integration of activity in starfishes have been discussed in a recent review (Smith, 1945). The present paper, on the kinematics of tube foot activity, is intended to serve as an introduction to a

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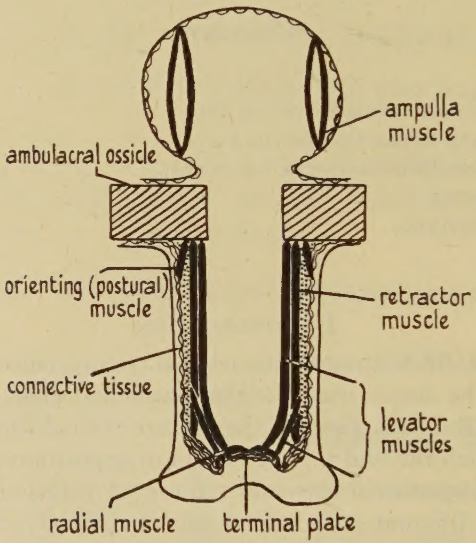
more detailed examination of the mechanisms underlying nervous integration in the starfish as evidenced by an analysis of the circumstances of stimulation and innervation appropriate to the appearance of the reflexly and centrally controlled activities of the feet.

II. The Movements and Postures of the Feet

The activities of a starfish podium comprise a number of readily recognizable movements and postures all of which originate in the contraction of smooth muscle fibres contained within the wall of the foot and of its annectant ampulla. Text-fig. 1, which illustrates schematically the arrangement and distribution of the principal systems of fibres, shows two series of muscles, the vertically running circumferential fibres of the ampulla and the longitudinal retractor muscles of the column of the podium, which have long been recognized (Greeff, 1871). The figure includes, in addition, three muscle systems not previously figured, namely, the basal sheath of orienting fibres, the radial fibres of the terminal plate, and the levator fibres of the plate, while omitting, for reasons later to be given, the musculature of the sucker figured and described by Cuénot (1891) and Chadwick (1923).

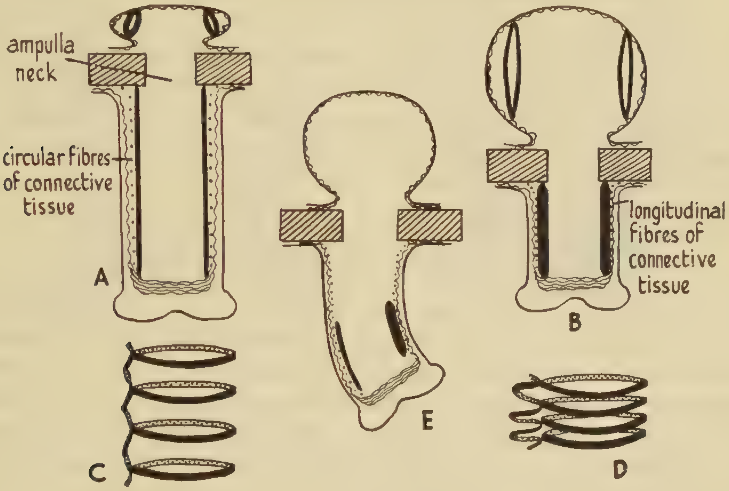


Text-fig. 1. Diagram of a longitudinal section through a foot and ampulla of *A. rubens* showing the arrangement of the principal muscle systems.

The muscles are separated from the fluid-filled cavity of the foot and ampulla by a thin coelomic epithelium and are bounded externally by a layer of connective tissue, to the collagen fibres of which they are attached either along their entire length or at their extremities. When the muscles contract, they initiate movements of the foot either by pulling directly on its wall or by exerting pressure on the fluid contained within the virtually closed system of the foot and ampulla. It will be the purpose of this account to define the particular muscle systems responsible for each activity and to examine the part played by antagonistic muscles, the hydrostatic skeleton, and the connective tissue sheath in the shaping of each movement and posture of the foot.

III. Protraction and Retraction of the Foot

Only brief reference will be made to these movements as their kinematics have been discussed in some detail in a previous paper (Smith, 1946).


ampulla
neck
circular fibres of connective tissue
A
longitudinal fibres of connective tissue
B
E
C
D

Text-fig. 2. a, b, and e. Diagrams showing the conditions of contraction and relaxation of the ampulla muscles and the retractor fibres of the foot during (a) protraction, (b) retraction, and (e) localized bending of the podium. Muscles in contraction are represented by the thicker, and relaxed muscles by the thinner, of the black bands. c and d show how the longitudinal and circular fibres of the connective tissue sheath within the column of the foot are arranged when the foot is (c) protracted, and (d) retracted.

Protraction (Text-fig. 2a) is initiated by the contraction of the ampulla muscles whereby fluid is expelled through the neck of the ampulla into the cavity of the podium. The inner layer of the connective tissue sheath of the foot comprises circular fibres (Text-fig. 2a), which, being inextensible, resist the pressures exerted on the side walls of the foot by the contained fluid and prevent a lateral bulging of the column; the effective pressure of the fluid is exerted against the terminal plate and the foot protracts. During protraction the longitudinal fibres of the outer layer of the collagen sheath, which in sections of a retracted podium (Text-fig. 2d) are seen to be much convoluted, gradually straighten; though at the normal limit of protraction (Text-fig. 2c), determined in the first place by the fluid content of the ampulla, they are still somewhat folded. By pulling at the foot it is possible to extend it farther, but attempts to stretch it beyond about twice its normal protracted length invariably result in the rupture of the wall of the column. In view of the observed degree of folding of the longitudinal fibres at various extensions of the foot it seems reasonable to infer that amputation occurs at the point when the longitudinal fibres are fully tautened.

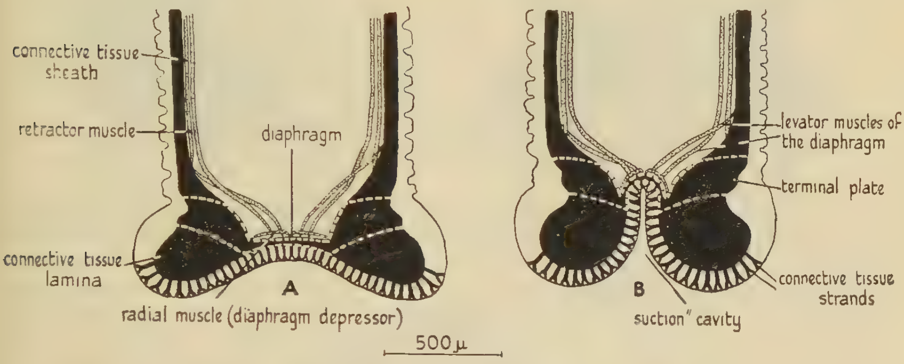
Withdrawal of the foot is brought about by the contraction of the muscles of the retractor sheath, fluid being expelled from the diminishing cavity of the foot into the ampulla, the muscles of which relax. So long as the conditions of retraction are comparable around the entire periphery of the sheath the foot retains its cylindrical form (Text-fig. 2B), but if there is differential contraction of the individual fibres, the foot bends towards the more contracted side (Text-fig. 2E). Localized bending movements of this kind are invariably exhibited by feet during their withdrawal from the substratum at the end of the backward step (Text-fig. 7, A4). In this instance the curvature arises as a result of the difference in length of the posterior and anterior faces of the podium (relative to the direction of the step): the former, being under greater tension at the moment of release, becomes concavely curved. As will later be shown, localized bending movements may also be evoked by unilateral stimulation of the foot column.

IV. Attachment and Detachment of the Sucker

Mucus undoubtedly plays an important part in the adhesion of starfish podia to solid surfaces, but the principal adhesive force in feet possessing a terminal sucker is provided by suction (Paine, 1926). Niemiec (1885) and Preyer (1886) attributed the suction effect to the cupping of the centre of the disk with a consequent lowering of the pressure within the fluid of the suction cavity. Whereas there has been general agreement on this point there is less unanimity concerning the disposition and role of the muscle systems by which the suction mechanism is operated.

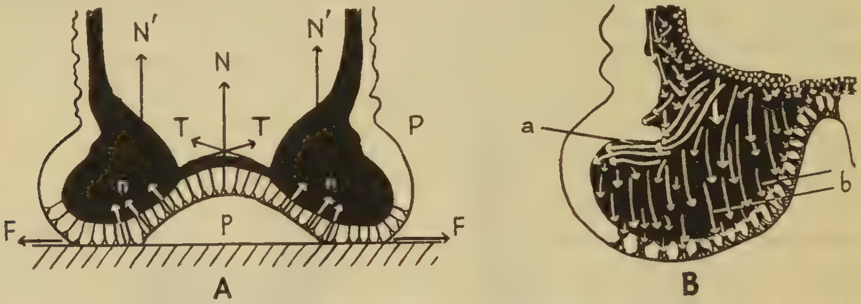
In 1891 Cuénot described and figured a system of radially arranged muscle-fibres within the sucker of the foot of *A. rubens*, distal to the terminal plate, and the view has been expressed (Sedgwick, 1927) that these muscles, by their contraction, cup the disk and fix the sucker. Notwithstanding Chadwick's (1923) confirmation of Cuénot's account, it is certain, however, that these radial fibres are not muscle but connective tissue. In sections stained with Heidenhain's iron-alum haematoxylin or with Delafield's haematoxylin and eosin, they may easily be mistaken for muscle, but when care is taken to differentiate myosin from collagen by the use of polychrome dyes such as Mallory's triple stain it is evident that muscle-fibres are absent from the disk and that the suction mechanism is operated by muscles wholly extrinsic to the sucker.

Text-figs. 3A and 3B show the principal systems of muscle-fibres and the disposition of the collagen connective tissue as seen in a median sagittal section of an *Asterias* foot, the material used being fixed in Heidenhain's 'Susa' mixture and stained in Mallory's triple stain. Although many of the fibres of the retractor musculature have a distal termination on the side wall of the podium, some of them (the levator muscles of the diaphragm) converge towards and are attached to the central area of the terminal plate of connective tissue. The upper and lower limits of the plate are indicated in the figure by the broken white lines and it will be seen that the centre of the plate (the diaphragm) is very much thinner than is its margin. The latter forms a thickened rim continuous, proximally, with the cylindrical connective tissue sheath investing the cavity of the foot. Distally, it is drawn out into a series of vertical laminae which thrust into the substance of the sucker as a series of radially arranged wedges, the arrangement of which is well seen in transverse sections through the sucker (Text-fig. 5A). Along a considerable part of the free margin of each lamina, and over the distal surface of the centre of the terminal plate, the connective tissue is frayed out into numerous strands (Text-fig. 3B), the individual fibres of which interdigitate with, and are attached to, the ectodermal cells of the sucker. When the muscles which are inserted on the upper surface of the diaphragm contract they cause it to be invaginated into the hydrocoel. The doming of the plate is clearly seen in sections through fully retracted, but unattached, feet (Text-fig. 3B). In this instance the tensions developed in the contracting musculature and transmitted through the collagen fibres of the sucker have caused a slight inward and upward rotation of the two laminae and a perceptible reduction in the diameter of the suction disk. A drawing-in of the disk is not, however, to be observed in feet the suckers of which are in contact with the substratum at the time of contraction of the retractor musculature.



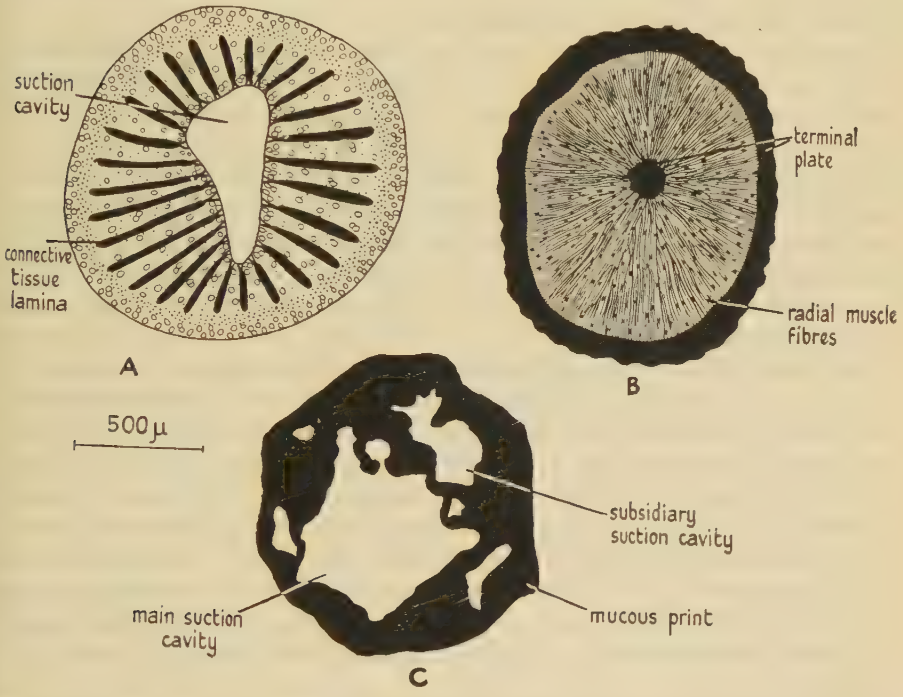
TEXT-FIG. 3. Diagrams of median sagittal sections through the distal end of (A) a protracted, and (B) a retracted, unattached, podium of *A. rubens*. The outlines of the foot and the distribution of the connective tissue (indicated by the black shaded areas) are, in each instance, accurately represented. Muscles are figured schematically.

Under these latter conditions (Text-fig. 4A) the levator fibres of the diaphragm exert a tension with a component (N) normal to its surface. The consequent tendency for the volume of the suction cavity to increase results in the pressure (p) within its contained fluid falling below the level of the pressure (P) acting on the outer wall of the foot. Suction will occur provided that the lateral wall of the cavity resists the tendency to collapse under the reduced pressure and provided also that the attached margin of the sucker does not slip inwards. It is, of course, evident, in view of the fact that suction does occur when the levator fibres pull on the terminal plate, that there is neither slipping of the foot nor a collapse of its wall, but the interest of the mechanism lies in the manner in which the properties and arrangement of the muscles and connective tissue ensure the rigidity of the wall during suction.



TEXT-FIG. 4. A. Diagram of a median sagittal section through the distal end of a tube foot of *A. rubens* showing the distribution and direction of the forces acting on the wall of the suction cavity during the attachment and detachment of the sucker. (*p*) is the pressure within the fluid of the suction cavity, (*P*) the pressure outside the foot. B. A part of the same section showing the orientation of the collagen fibres within the terminal plate and one of the radial laminae of the sucker. In both figures the connective tissue is shown in black. (*a*) the horizontally oriented fibres of the outer margin of the lamina; (*b*) the longitudinally oriented fibres terminating on the distal and inner edges of the lamina.

The tendency of the margin of the sucker to slip inwards is resisted by centrifugally acting frictional forces (*F*) and by the horizontally and centrifugally acting components of the forces (*n*). These latter forces are the tensions developed in the connective tissue laminae of the sucker as a result of the contraction of the retractor muscles *N′ N′* inserted around the periphery of the terminal plate. The terminal plate and the laminae are composed of a network of collagen fibres which have the property of being inextensible when subjected to forces applied along their length. While (Text-fig. 4B) the proximal face of the terminal plate is made up of closely matted circumferential fibres—in effect the most distal of the circular fibres of the inner zone of the cylindrical sheath of the column—the wedge-shaped laminae are composed of fibres which have a predominantly longitudinal orientation. As will be seen from Text-fig. 4B, some of the fibres (*a*) curve outwards to form the thickened margin of the upper and outer surface of the lamina, but for the most part (*b*) they run downwards while turning all the while towards the midline of the foot. The result of this arrangement is that by far the greater number of the fibres converge on the distal and inner margins of the lamina, to be inserted at right angles to its surface. It is along a length of the lamina exactly corresponding to the line of normal insertion of the main series of fibres that the collagen tissue is frayed out into the branching strands which ultimately terminate between the cells comprising the wall of the adhesive disk and suction cavity. The inextensibility of the collagen fibres, their orientation normal to the wall of the cavity and disk, and the arborescent endings of the system collectively contribute to a mechanism that is admirably adapted for translating, with maximum effect, the tensions (N') developed in the contracting muscles of the retractor sheath into forces (n) acting outwards from all parts of the lateral wall of the suction cavity. The rigidity of the wall of the cavity being thereby assured, the suction mechanism can operate on the principle of a piston (the central diaphragm of the terminal plate) tending to be drawn up within a rigid cylinder (the margin of the sucker).



TEXT-FIG. 5. A and B. The structures seen in transverse section of the distal end of a foot of *A. rubens*. A. Below (distal to) the terminal plate. B. Through the upper (proximal) surface of the terminal plate. C is a drawing of the mucous print of an attached sucker. The area of contact of the sucker with the substratum is shown in black.

Mucous prints left by the suction disk (Text-fig. 5C) show that, in addition to the main suction cavity, there are a number of subsidiary spaces functioning as secondary centres of negative pressure. As will later be shown (p. 8), they may in addition play an important part in effecting the detachment of the disk.

The primary condition of detachment of the sucker is that the forces tending to hold it to the substratum shall be opposed by greater forces acting in the opposite direction. Forces tending to pull the foot away from the substratum may be generated within the foot itself or be applied externally by the traction of the animal as a whole as, for example, during locomotion. As we have seen, however, the negative pressure of suction will hold against these traction forces beyond the point of rupture of the column of the foot and of amputation of the podium. Before a foot can be withdrawn undamaged from the substratum its disk must first cease to act as a sucker.

Absence of suction implies that the pressure within the fluid of the suction cavity has become equilibrated with the pressure acting on the outer wall of the disk. There appear to be two possible ways of effecting this change: (1) by allowing the outside sea-water to communicate with the fluid of the suction cavity, and (2) by the exertion of a pressure on the walls of the suction cavity equal to the negative pressure of suction.

It will be seen on examining a sucking disk prior to and at the moment of withdrawal of the foot from the substratum that, on occasions, the margin of attachment exhibits a slight wrinkling. One effect of this wrinkling will be to enlarge some of the subsidiary cavities of the suction disk and to permit of their communication one with another. If the water channels so formed lengthen sufficiently in a radial direction they will ultimately open both to the outside of the disk and into the central cavity and the disk will no longer be able to adhere by suction. The frequent detachment of the foot immediately after wrinkling has occurred appears to offer direct evidence of the actual operation of this mechanism.

It is, however, more usual for sucking disks to become detached without the occurrence of any previous wrinkling. In this event the walls of the suction cavity have presumably been subjected to forces tending to press them inwards and so to increase the pressure within the contained fluid. As will be seen by reference to Text-figs. 3 and 4A, there are no mechanisms by which an inwardly acting pressure can be exerted on the lateral walls of the cavity. These walls, solidly built and firmly attached at their base to the substratum, will not, moreover, yield to centrifugally acting forces such as will be developed when the pressure within the suction cavity rises. In the detachment, as in the attachment of the sucker, they therefore remain relatively immovable and rigid.

The central diaphragm of the terminal plate, forming the roof of the cavity, is, however, in different case. A thin circular disk with a slight convexity into the hydrocoel, it has attached to its upper surface a number of radially arranged muscle-fibres (Text-figs. 3A, 5B, radial muscle) which, originating on the inner margin of the thickened rim of the terminal plate, are inserted at the centre of the diaphragm. It will be evident that the whole relations of these radial muscles, their limitation to the one part of the wall of the suction cavity that is capable of being distorted, their radial arrangement, and their origin on a relatively immovable plate, are such as to ensure that, on contracting, they will exert forces acting tangentially to the surface of the diaphragm (Text-fig. 4A (*T*)) and so, by tending to depress the diaphragm, will cause a rise in pressure within the fluid of the suction cavity and the eventual cessation of suction.

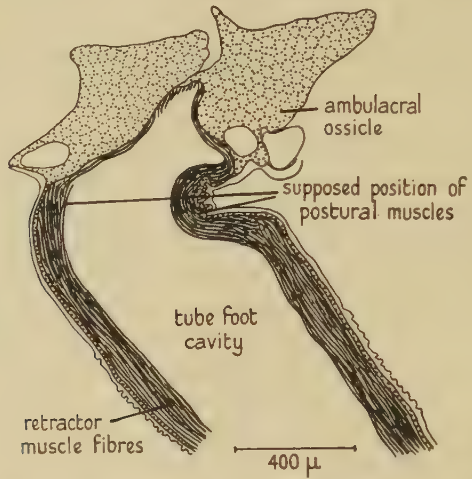
The two methods of release from suction, by a wrinkling of the sucker and by the exertion of tangentially acting forces on the surface of the central diaphragm of the terminal plate, are both brought about by the contraction of the radial muscles. Attachment of the sucker results from the contraction of the longitudinally oriented levator fibres of the diaphragm. Consequently these two systems of muscles are functionally antagonistic. Since, however, they may at times be in simultaneous contraction, as, for example, at the moment the sucker is pulled away from the substratum, they cannot be regarded as standing solely in the relation of reciprocally contracting and relaxing systems. It is interesting in this connexion to note, as will be shown in a later communication, that they are innervated through different systems of nerve arcs. There is accordingly no *a priori* reason why they should always act reciprocally.

V. POSTURAL POINTING

These activities include all postures of the feet that can be said to be oriented in the sense that the podia, instead of hanging vertically downwards from the under surface of the arm, have their long axes directed obliquely to the surface. Postural pointing is exhibited during the various phases of the ambulatory step (Text-fig. 7B, 1–4), and in lateral pointing, in which latter movement the foot swings directly outwards from the mid-line of the arm.

Hamilton (1922) alone of previous authors has considered the implications of these postures in terms of the muscular anatomy of the foot. He found that the distal part of the column of a starfish foot can be twisted without affecting the angle of orientation of the podium, and concluded from this that the orienting musculature must lie towards the base of the foot. One doubts whether it is possible to draw this conclusion from a repetition of Hamilton's experiment on the foot of *A. rubens.* The podium, if allowed to attach to a needle, can be twisted readily enough but its orientation, while so attached, is too much influenced by external forces for any assessment to be made of the freely developed angle of orientation. Moreover, as soon as the needle is removed, the foot withdraws and its former pointing attitude is lost.

The occurrence of a basal orienting musculature can, however, be justified on other grounds, in spite of the fact that it is not to be distinguished in sections of the foot as an anatomically distinct system of fibres. When longitudinal sections through a foot, killed and fixed in the pointing position, are examined (Text-fig. 6) it is seen that the side of the podium which makes an acute angle with the under surface of the arm is not curved but is wrinkled at its base. These circumstances suggest that pointing results from the unilateral contraction either of short fibres within the base of the foot or of the most proximal parts of the long fibres of the retractor sheath. The latter hypothesis leads to evident difficulties. It necessitates, for example, the supposition that, during the change from the retracted (Text-fig. 7, B1) to the protracted pointing position (B2), the basal part of each of the protagonistic fibres remains contracted at a constant length while its more distal portion undergoes extension. Or again, it requires that, during the backswing of the foot (Text-fig. 7, B2 to B3), the proximal parts of the protagonistic and antagonistic fibres shall undergo reciprocal contraction and relaxation while maintaining a substantially unaltered length distally. While such sequences and combinations of accurately graded differential contractions within the limits of a single fibre, applying *mutatis mutandis* to all the fibres of the ring, are not beyond the bounds of possibility, it seems more reasonable to suppose, in view of all the circumstances, that the postural and retractor muscles not only function as, but are indeed, in actual fact, morphologically separate systems.



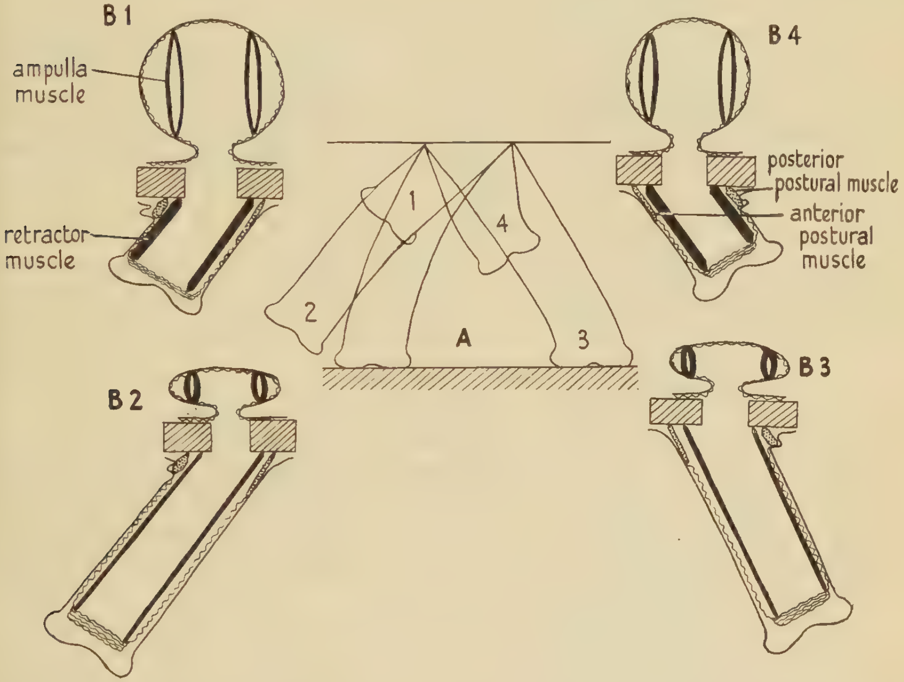
TEXT-FIG. 6. A longitudinal section through the base of a pointing foot of *A. rubens*.

It must further be supposed, since the foot can be directed towards any point of the compass, that the orienting muscles, like the retractor fibres, comprise a cylindrical sheath which, in this instance, consists of short fibres encircling the base of the podium.

VI. LOCOMOTORY STEPPING

During locomotion all the ambulatory feet step in the line of advance of the starfish. The form of the locomotory step is shown in Text-fig. 7A. A retracted podium first orients with its tip thrust forward (A1). The foot then protracts and, when fully extended (A2), swings back through an angle of about 90° with the sucker pressed against, but not firmly attached to, the substratum. This pendulum-like movement has the effect of thrusting the base of the foot forward relative to the sucker, and the sum of the forward thrusts of all the stepping feet determines the movement of the animal as a whole. At the end of the backswing (A3) the sucker detaches and the foot is withdrawn. During the withdrawal movement the foot re-orientates so as again to point in the forward direction (A1).

The step is thus a cyclical activity, comprising a series of linked movements. Ideally it should consist of four movements—protraction, swing back, retraction, and swing forward—alternating with four transitory phases of static posture (Text-fig. 7, B1–B4). Each posture would be characterized by the static contraction of one member of each of the two opposing pairs of muscles engaged in the execution of the step, namely, the protractor–retractor series and the diametrically opposed protagonists and antagonists of the orienting ring, the four postures representing the four possible combinations of action of the two pairs of opposing muscles. These conditions are set out in Table I below, muscles in contraction being represented in the larger, and relaxed muscles in the smaller, type.



TEXT-FIG. 7. A1–A4. The successive phases of the ambulatory step. B1–B4 show the conditions of contraction and relaxation of the protractor, retractor, and postural muscles of the foot during the successive phases of static posture of the 'ideal' step; the protractor and retractor muscles are shown in black, the orienting (postural) fibres are stippled. The anterior postural fibres orient the foot in the forward direction, the posterior fibres in the backward direction of the step.

As the arrows in Table 1 indicate, each phase of static posture ends, and a new movement begins, with a reversal of the contraction–relaxation relationships of one of the two pairs of opposing muscles, the stepping cycle comprising, in its ideal form, a regular alternation of such changes, first one and then the other pair of muscles being affected.

The actual locomotory step departs from the idealized cycle in certain particulars. As has already been noted, the phase of posteriorly oriented protraction (Text-fig. 7, B4) is omitted: the foot in passing directly from phase 3 to phase 1 combines the movements of withdrawal and of anterior orientation.

TABLE I. *Static phases of the ‘ideal’ step**

Anteriorly oriented retraction. Phase B1.	Anteriorly oriented protraction. Phase B2.	Posteriorly oriented protraction. Phase B3.	Posteriorly oriented retraction. Phase B4.
ANTERIOR POSTURAL M. Posterior postural m.	ANTERIOR POSTURAL M. Posterior postural m.	Anterior postural m. POSTERIOR POSTURAL M.	Anterior postural m. POSTERIOR POSTURAL M.
RETRACTOR FOOT. Protractor foot (Ampulla m.).	Retractor foot. PROTRACTOR FOOT.	Retractor foot. PROTRACTOR FOOT.	RETRACTOR FOOT. Protractor foot.

Protraction B1–2	Swing back (Effective step) B2–3	Withdrawal B3–4	Swing forward B4–1

Phases of movement of the ‘ideal’ step.

In the actual step.

Tendency for protraction to be delayed.	Marked tendency for swing back to be delayed.	Withdrawal and swing forward occur simultaneously. Phase B4 omitted.

* The phases of static posture B1–B4 correspond to B1–B4 of Text-fig. 7. Movements are represented by their initial and terminal postures, e.g. B1–B2.

Evidently, at this time, two sets of muscles—the retractor muscles of the foot and the anterior postural muscles—are in simultaneous contraction. Secondly, the smooth rhythm of the step is liable to become dislocated. There is a tendency for the foot to remain poised, often for some seconds, either in the position of anteriorly oriented retraction (B1), or (more frequently) of anteriorly oriented protraction (B2), the podium hesitating either to begin its movement of protraction or to initiate the backswing. These three modifications of the idealized cycle are explicable on the assumption that in each of the two series of opposing muscles one member of each pair is more readily excited to contract than is its partner. Thus, in the protractor–retractor series, the foot muscles are more readily excited than are those of the ampulla while, in the orienting ring, the anterior fibres respond more readily than their posterior antagonists. Some implications of these events will be discussed in a later paper when the nervous mechanisms underlying stepping activity are examined.

ACKNOWLEDGEMENTS

It is a pleasure to record my thanks to Dr. C. F. A. Pantin, F.R.S., and to Dr. R. H. J. Brown, both of whom have offered suggestions and criticisms of which I have been glad to take advantage.

SUMMARY

1. An account is given of the muscular anatomy of the foot and ampulla of *Asterias rubens*. An intrinsic musculature of the sucker figured by Cuénot (1891) and Chadwick (1923) is shown not to be present; on the other hand, postural muscles responsible for orientating the podium, levator fibres which 'cup' the sucker, and radial fibres which flatten it are described and figured for the first time.

2. The role of the different muscle systems, the collagen connective tissue, and the fluid of the hydrocoel in protracting and retracting the foot, and in effecting the attachment and detachment of the sucker, is discussed.

3. Evidence is presented to show that postural pointing of the foot is brought about by the contraction of a ring of muscles encircling the base of the podium. The orienting muscles are functionally, but not anatomically, distinct from the longitudinal fibres of the retractor sheath.

4. The ambulatory step is shown to comprise a series of linked phases of static posture and of movement. Each phase is characterized by the contraction of one member of each of the two opposing pairs of muscles engaged in the development of the step. The two pairs of muscles are (1) the anterior and posterior orienting fibres, and (2) the protractors and retractors of the foot. In its ideal form the step comprises four phases of static posture alternating with four movements. Each movement is ushered in by a reversal of the contraction–relaxation relationships of one of the two pairs of opposing muscle systems. Four such changes are possible and they occur in a sequence that ensures the orderly succession of the four movements of protraction, swing

back, retraction, and swing forward, of which movements the idealized stepping cycle is composed.

5. The actual locomotory step departs from the ideal form in two respects: (1) it is liable to become disrupted by a delay in the initiation of the protraction or of the backswing movement, and (2) withdrawal of the podium occurs simultaneously with its re-orientation in the forward direction. It is pointed out that these variations are explicable on the assumption that, in the two series of opposing muscle pairs, the retractor fibres are more readily excited to contract than are their antagonists, and the anterior postural muscles than the posterior postural fibres.

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The Nephridia of *Geonemertes dendyi*

BY

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With five Text-figures

GEONEMERTES DENDYI (Dakin) is a terrestrial nemertine. The genus has a predominantly Australian distribution, but in recent years this species has been recorded from the western parts of England and Wales (Waterston and Quick, 1937; Pantin, 1944). In this genus the nephridial system is highly developed, and its structure in several species has been described by Hett (1924), Coe (1929), and others. Stammer (1934) mentioned the existence of a nephridial system in *G. dendyi* but did not describe it. The present work arose from a study of this species collected in South Devon, in all stages from the newly hatched young to the adult. The nephridial system proves to be at least as highly developed in this as in other species, and there are points of some interest regarding its function.

METHODS

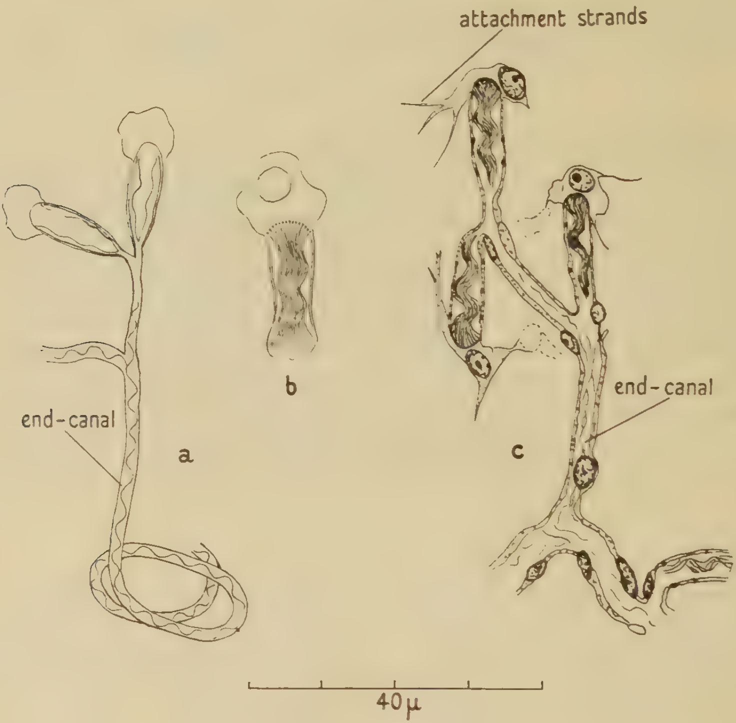
The flame-cells and the proximal part of the tubule system into which they open are easily detected in living worms owing to their ciliary activity, particularly in newly hatched larvae (about 1 mm. long) and immature specimens of about 3–4 mm. in length. The distal parts of the tubule system show no signs of ciliary activity and have not yet been observed in life.

The system is best seen if the worms are immersed in tap-water and slightly compressed with a large coverslip, or held by a small square of wet cellophane covering a shallow hanging drop on the underside of a coverslip above a moist chamber. For prolonged observation under a powerful light, absorption of heat-radiation entering the condenser is essential.

For micro-anatomical studies the best fixatives were found to be Susa, and Zenker in which formic acid is substituted for acetic. Brief preliminary anaesthetization with 5–10 per cent. ethyl alcohol had no apparent ill effects and enabled the specimen to be fixed without distortion. This was done by arranging the animal on a waxed coverslip, covering it with a piece of moist cigarette paper, and dropping it face down on the fixative. Convenient stains were Mallory's Triple Stain, 'Azan', and Heidenhain's Iron-Haematoxylin with or without Masson's Ponceau-Light Green used as counterstain. Paraffin sections 4μ–10μ were cut, commonly after Peterfi's celloidin-paraffin method.

STRUCTURE

In general the nephridia of *G. dendyi* conform to the plan described by Hett (1924) in *G. hillii*, and by Coe (1929) in *G. agricola*. The flame-cells are exceedingly numerous beneath the dermal musculature. The terminal ducts from groups of them unite in a common canal, the 'end-canal', which passes to a glandular tubule from whence goes a final efferent duct to one of a very large number of external openings of the system, scattered all over the body.



TEXT-FIG. 1.

A. Living flame-cells and end-canal with helical ciliary wave. B. Isolated active flame-cell from squashed specimen, showing basal granules and partial fraying of end of ciliary flame. C. Flame-cells and end-canal: Formic-Zenker and iron haematoxylin.

Text-figs. 1 A, B, and C illustrate living and fixed flame-cells of *G. dendyi*. In fixed material the terminal chamber is 12–17μ long by 3·5μ broad, thus being slightly smaller than those of *G. agricola*, but decidedly larger than those in *G. hillii*. Active living flame-cells are considerably broader than fixed ones, averaging about 17μ by 4·5μ. Though it is difficult to measure them alive except during activity, their size seems to diminish somewhat when the ciliary flame stops moving, and their smaller size when fixed may not therefore be entirely an artifact of fixation.

The head of the cell is occupied by a mass of protoplasm which in fixed material appears to be attached to neighbouring structures in the parenchyma by two or three fine strands. As in *G. hillii*, but unlike *G. agricola*, there is only one nucleus in the head of the cell and none in the wall of the terminal chamber. Indications of two or three thickened bands may be seen encircling the terminal chamber, but they do not seem to be as evident as those in *G. agricola*.

In life the terminal chamber is somewhat flattened in a plane parallel to the surface of the worm. Within this tube and lying in the same plane is the powerful tongue-like ciliary flame. Like the flame itself, the sides of the chamber are roughly parallel until near the tip, where they converge to its end-canal.

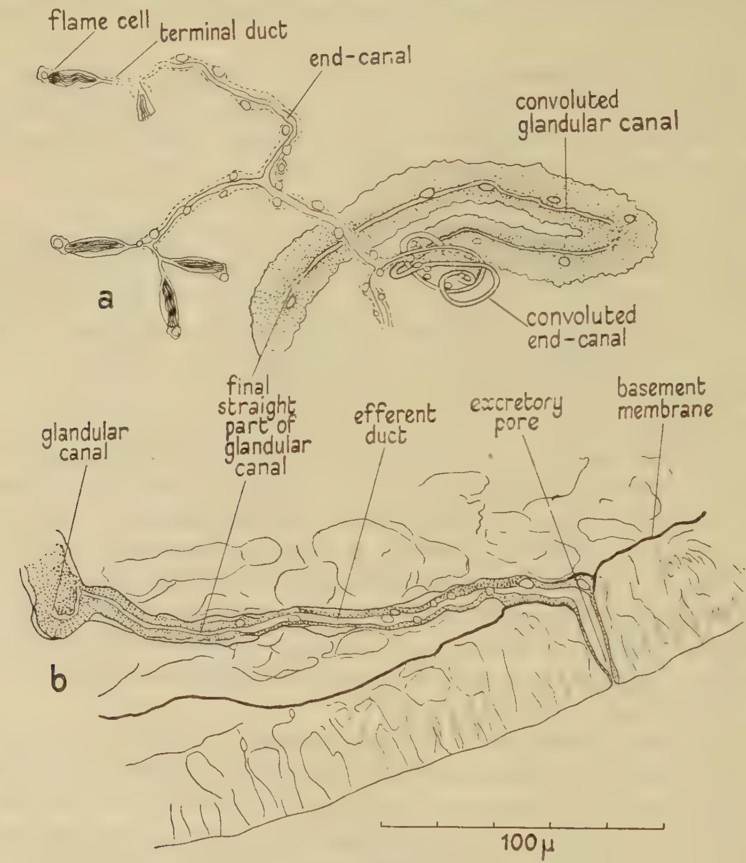
A clearly defined cushion of protoplasm caps the head of the chamber. Along this cap is a row of basal granules from whence arise the long individual cilia which compose the flame (Text-fig. 1 B). In life the cilia are fused together, though they gradually separate under adverse conditions, a ragged separation first appearing at the tip. The living 'flame' is highly refractile and easily seen.

During activity, sinusoidal waves of contraction pass down the tongue-like flame with a frequency which varies from just above the flicker rate (*c.* 10 per sec.) down to about two a second. At any moment, two to three complete waves are in progress along the flame, their amplitude being about half a wave-length. Under conditions to be described later, the flame may gradually stop. As it does so, the frequency falls off without any change in wave-length or at first in amplitude. On cessation of the beat, the waves may be left for a moment 'standing' and then disappear, leaving the flame extended. As this condition is approached, waves may cease to be propagated along the whole length of the flame.

The active flame almost fills the terminal chamber when viewed in breadth. Viewed sideways, the flame does not fill the chamber when at rest, but during activity the crests of the waves seem to do so, a fact of importance in considering the mechanical efficiency of the flame-cell. At rest, the flame extends straight down the chamber, as it often does after fixation in 70 per cent. ethyl alcohol. Fixation in Susa and Zenker leaves the flame in wave form, often exaggerated.

The staining reactions of the flame in some ways resemble those of the nearby muscle-cells rather than those of the cilia of the ectoderm. It has a strong affinity for acid fuchsin, and the dark colour of iron haematoxylin is retained nearly as long as in the muscle during differentiation.

Text-fig. 2 shows the relation of the flame-cells to the rest of the nephridium. The flame-cells are usually in pairs, the terminal ducts of which join after a short distance (Text-fig. 1). A branched end-canal receives the ducts of several pairs and ultimately passes into a glandular duct. Terminal ducts and end-canals have moderately thin walls with scattered nuclei but without apparent cell-boundaries. In *G. agricola* the end-canals open directly into the glandular part of the nephridial duct, which is convoluted (Coe, 1929). In *G. hillii* they are in addition frequently coiled (Hett, 1924). In *G. dendyi* the end-canals appear to be more highly organized. Branches from the terminal ducts of the flame-cells unite to form a canal which commonly runs fairly straight for a considerable distance. As it does so the canal widens from about 2 μ to 4 μ in diameter, and the walls thicken slightly. The canal then undergoes two or three convolutions, after which it passes into a thick-walled glandular part of larger cross-section but of rather smaller lumen. This glandular part generally continues the convolution (Text-fig. 2 A). The convoluted portions of both end-canal and glandular duct are contained in well-defined spaces in the parenchyma. During life the end-canal is easily seen owing to the ciliary movement. This is absent in the terminal ducts. The movement begins in the branches of the end-canal and becomes stronger as

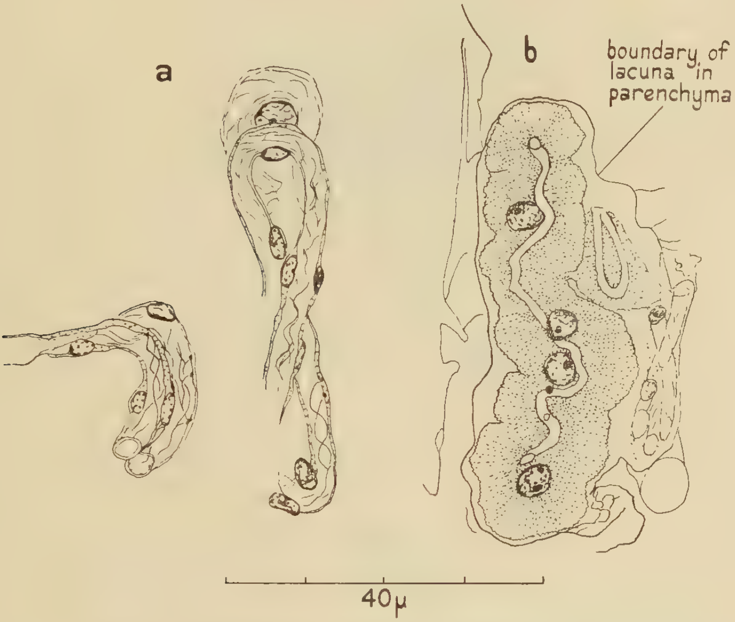


TEXT-FIG. 2. Camera lucida outlines.

A. Flame-cells, end-canal with convolution, glandular canal.
B. Glandular canal, efferent duct, excretory pore.

the convolution is approached. Ciliary movement in the convolution presents a striking appearance. It seems to finish abruptly at about the point where sections show the glandular region to begin. Fixed material shows that the lumen of the end-canal carries long fine helical fibrils, apparently cilia (Text-fig. 3 A).

The action of the cilia gives the appearance of helical waves running rapidly down the end-canal and round its convoluted portion. That the motion is helical, and not a plane wave as in an undulating membrane, is apparent from the fact that it can be seen from whatever angle the canal is viewed. The appearance (Text-fig. 1 A) resembles that seen in the nephridial ducts of Lumbricus.



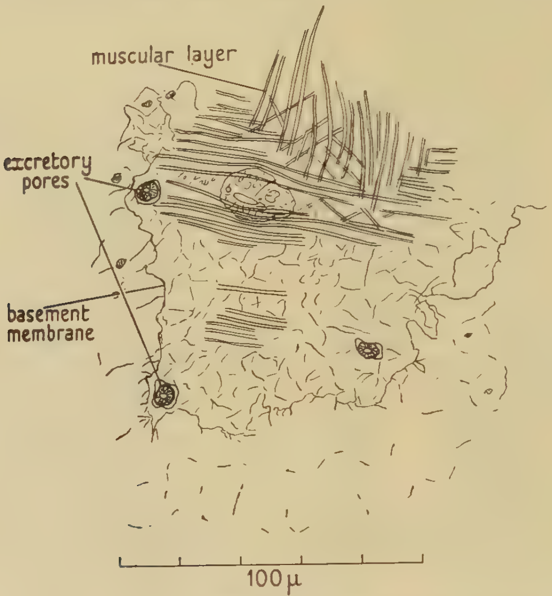
TEXT-FIG. 3. Camera lucida outlines.

A. Convoluted end-canal with cilia. B. Part of glandular canal in lacuna of parenchyma.

No cilia can be seen beyond the convoluted end-canal in life, and none can be detected in sections of the glandular canal or efferent duct to the exterior. The walls of the glandular canal are thick and granular and have the well-marked radial structure recorded by authors in other species (Text-fig. 3 B). In poorly fixed material the radial fibres actually separate, giving the tubule the appearance of a contorted bottle-brush. As in other species, there are scattered nuclei, but no cell boundaries are evident. Evidence will be presented later that the end-canal with its convolution and the glandular canal are physiologically distinct.

The glandular canal finally leaves the lacuna of the parenchyma occupied by the convolution, and may run some distance in a fairly straight path

(Text-fig. 2 A and B). The histology of the duct then changes again to that of a thin-walled tube. This, after running just beneath the basement membrane, abruptly enters one of the numerous excretory pores. These consist of a hollow flask-shaped cell resting on the basement membrane and opening to the exterior by a minute pore (Text-fig. 2 B). How numerous these pores are is shown in Text-fig. 4, taken from a tangential section through the dorsal surface and basement membrane. Over much of the surface they are only 50–100 μ apart.

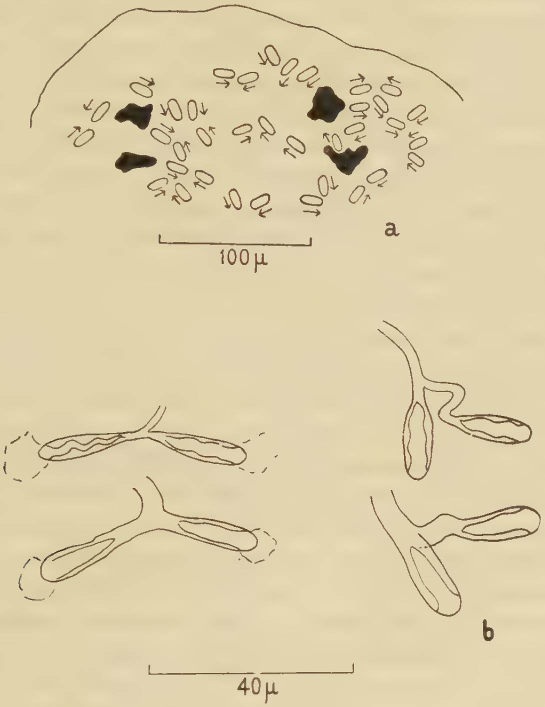


TEXT-FIG. 4. Tangential section through dorsal surface.

ANATOMICAL RELATIONS

The body of *G. dendyi* differs from that of most marine metanemertines in the thinness of the muscular body-wall and the voluminous parenchyma. The latter consists largely of connective tissue divided into well-defined lacunae and channels containing structures such as the convoluted tubules of the nephridia and the blood-vessels. It is this tissue which varies most clearly in volume according to the water content of the animal.

The thin muscular layer lies immediately below the basement membrane, and among and immediately below the innermost muscle-fibres, the longitudinal ones, lie the flame-cells. In *G. dendyi* they are confined to this plane and do not seem to occur in other situations, such as below the surface of the rhynchocoel. They are most numerous over the head and along the sides of the body. They are fewer along the mid-ventral line, and fewest along the mid-dorsal line, above the rhynchocoel. Text-fig. 5 A shows the distribution of flame-cells on the upper surface of a newly hatched (1 mm.) larva. As the animals grow, the relative number of flame-cells increases even more rapidly, so that over the head and sides no point on the surface of the animal is more than 10–20μ from one of these structures. Water entering or leaving the body must pass near one or more of them.



TEXT-FIG. 5.

A. Head of immature larva showing distribution of active flame-cells: arrows show direction of flame. B. Sketches of flame-cells showing swelling of terminal ducts after compression.

Below the muscular layer and among the nephridia there ramifies a network of fine contractile blood-vessels, derived from the lateral vessels. As Coe (1929) points out in other species of Geonemertes, so also in *G. dendyi* the flame-cells are not in intimate contact with the vessels, as they are in some marine nemertines; they are, however, always near them and occupy the same stratum below the muscle layer.

The convoluted tubules in their lacunae of the parenchyma lie distinctly deeper than the flame-cell-vascular layer. The efferent ducts running out from these ultimately make their way again to the surface.

FUNCTION OF THE PROTONEPHRIDIA

Since the time when Goodrich (1899) made the suggestion that the solenocytes of *Glycera* are functionally analogous to the Malpighian corpuscles of the vertebrate kidney and excrete fluid, the analogy has been extended to other protonephridia, particularly in Platyhelminthes (Reisinger, 1922; Westblad, 1922). Apart from the fact that the flame-cell system of freshwater Turbellaria is much better developed than that of their marine relatives, there is direct evidence in various acoelomates that water is excreted by such systems. Water can be seen to accumulate in the contractile bladders of some Rhabdocoela, Trematoda, and Rotifera, and to be periodically emptied. Herfs (1922) showed that in certain cercariae the rate of water secretion decreased as the osmotic pressure of the external medium was increased.

In *G. dendyi* there is no doubt that the upper part of the protonephridial system secretes fluid. If a relatively transparent individual is left for a time in fresh water and then compressed under a coverslip, the flame-cells and the ducts leading immediately from them can sometimes be seen to swell during the course of 10–15 minutes (Text-fig. 5 B). This is most often to be seen in the flame-cells that are farthest from the edge of the animal, and are therefore the most subject to compression.

Observation shows that there is a relation between excretion of water and the activity of flame-cells. During life this is often intermittent, or the cells may even be inactive for long periods. No swelling of the tubule system has so far been seen except when the ciliary flame is active. Moreover, the activity seems to be related to the water-content of the internal medium. It is absent in animals that have been allowed partly to dry, but is resumed if the animal is immersed for a time in tap-water. If an animal showing intermittent activity of the flame-cells is examined under a coverslip, it is seen that local groups of the cells begin to be active more or less simultaneously. Later, all gradually stop, though some continue to beat many minutes longer than others. Renewed activity often promptly follows local movements in the worm, either contraction of the body or the peristalsis of a blood-vessel in the neighbourhood of the flame-cell. Immobility of the worm is always followed sooner or later by inactivity of the ciliary flames, except those on the edge where the body is in contact with water under the coverslip. Activity of the flames can often be engendered by slightly raising and lowering the coverslip—as when focusing an oil-immersion lens when the coverslip is unsupported. Thus anything which tends to stir the internal medium round the flame-cells leads to the activity of the flame, while stagnation of the medium is correlated with inactivity.

If an animal with active flames is carefully squashed under a coverslip, it is possible to reach almost complete disintegration of the tissues while the individual flame-cells still beat for a time. If when they cease to be active, tap-water is allowed to run under the coverslip, the flames restart—even though they sometimes appear to be more or less isolated from neighbouring cells and detritus. It is possible to revive the activity of the flame-cells in the squashed mass by irrigation several times in succession. It is even possible sometimes to revive them by lifting the coverslip with some adhering cell debris and flame-cells and remounting it in a fresh drop of tap-water. It is remarkable that the flame-cells should be able to resume or to continue their activity after breakdown and removal of much of the surrounding tissue.

DISCUSSION

It is difficult to avoid the conclusion that dilution of the internal medium of the parenchyma is a stimulus to the activity of the flame-cells. And since under conditions of activity water appears to pass into the nephridial system, it is natural to suggest, in conformity with common opinion, that the ciliary flame actually drives water into the system. But Carter (1940) has pointed out a difficulty in assuming the mechanism is powerful enough for this purpose. Ciliary and flagellar mechanisms elsewhere in the animal kingdom have not been shown to be able to produce large differences of pressure. Parker (1919) found the oscular pressure of the sponge *Stylotella* to be 3·5–4 mm. of water. Such pressures would be wholly inadequate for the separation of water from the salts in the body fluid. But they might suffice to overcome a very low colloidal osmotic pressure (cf. Picken, 1937), and Reisinger (1922) suggested that in the excretion of water by flame-cells the colloids of the body fluid might be kept back. The mechanical plan of a flame-cell seems to be far more efficient than that of the flagellate chamber of a sponge for the creation of a pressure-difference.

It has been suggested (Carter, 1940) that the cilia of flame-cells may not function as a pump, but rather as a stirring mechanism to prevent accumulation of substances which would hinder a process of active secretion. But since fluid is passing into the tubule system it seems unlikely that there could be such an accumulation on the inner boundary of the terminal chamber.

While it has not been possible to obtain a direct demonstration that the ciliary flame in *G. dendyi* is responsible for the flow of fluid into the tubule system, the observations recorded here certainly suggest this. Due weight must be given to the fact that if a ciliary mechanism can exert the necessary pressure-difference at all, the mechanical design of a flame-cell, as seen in operation, seems admirably adapted for such a purpose. Further, there is evidence that some pressure at least is set up by the action of the flame. If the tissues of an animal are gently squashed under a coverslip so as partly to separate the flame-cells from the surrounding tissues, the action of the flame sometimes causes visible oscillations of the base of the cell. Since the flame is apparently free inside the terminal chamber, the oscillation is presumably due to pressure-differences set up within the chamber.

Whatever the part played by the ciliary flame in the process, the evidence suggests that the nephridia of *G. dendyi* excrete water. But though it is easy to relate the development of the nephridia of freshwater acoelomates to an inevitable osmotic intake of water from the medium, the existence of a mechanism for excreting water in a terrestrial animal is remarkable. Such an animal might be expected to provide against loss of water rather than for its excretion. Loss of fluid in *G. dendyi* may certainly be great. The worms normally range from 1 to 20 mm. in length by 0·5 to 3 mm. in breadth. They continually secrete a tube of mucus in which the animal moves forward during locomotion, through the action of cilia which completely cover the body. Loss of mucus and evaporation cause the body visibly to diminish in size when exposed to an unsaturated atmosphere. When unable to escape to a moister place the animal ceases to move and the outer mucus hardens to a transparent cocoon in which water loss appears to diminish. Such cocoons, however, are sometimes formed to provide an aqueous medium for the eggs which are shed into them, together with a quantity of fluid. This is a further tax on water reserves, though the eggs are normally laid in very damp situations, where there may even be a film of running water. But the most rapid loss of water takes place when the proboscis of the worm is everted. This is primarily a flight response in this species, and when it occurs so much mucus is lost that after three or four eversions the animal becomes visibly smaller.

But while water loss may sometimes be considerable, it is incorrect to suppose that desiccation operates continuously. The worms live under damp stones and logs, particularly in woods where low vegetation impedes the drying action of the wind. They possess strongly developed responses, particularly positive geotaxis and negative phototaxis, which remove them from exposed situations. In a gradually drying vivarium they can be seen to bury themselves. Though sometimes to be found far from fresh water, they may be taken from under a log, of which the other end lies in a film of water sufficient to support *Planaria vitta*. They are often found in contact with dew or rain-water, and survive immersion in fresh water for some days, though not indefinitely. The worms are, in fact, frequently exposed to pure water: a situation more exacting than that to be met by many freshwater organisms. Their natural conditions of existence are thus not those of continuous desiccation, but may vary between this and excessive hydration. Hence any regulating mechanism must be able to deal with extremes of water conditions. The evidence presented here shows that the nephridial system can eliminate excessive water, and that under conditions of water loss the ciliary flames become inactive. What part is played by the highly differentiated portions of the nephridial canal remains to be seen.

I wish to express my gratitude to Mr. O. D. Hunt and Mr. B. W. Sparrow for much help in the collection of specimens.

SUMMARY

1. The nephridia of the terrestrial nemertine *Geonemertes dendyi* are described. The flame-cells are in groups, each of which communicates with the exterior by its own duct. The flame-cells open into the branches of a ciliated end-canal. Distally this end-canal executes a number of convolutions and then passes into a non-ciliated glandular canal. The first part of the glandular canal is also convoluted, and the distal part of it passes to the efferent duct.

2. The nephridia are distributed over most of the surface of the animal immediately below the muscular layer. The convolutions of the end-canals

and glandular canals lie somewhat deeper, in well-defined lacunae in the parenchyma.

3. Evidence is presented that fluid can be excreted into the nephridia. The activity of the flame-cells varies with the degree of hydration of the animal and may cease on desiccation. The flame-cells can be stimulated to activity by dilution of the body fluid of crushed animals with tap-water. The relation of water excretion to the mechanical activity of the flame-cells is discussed.

4. Some features of the natural habitat and the water relations of the animal under natural conditions are described. It is pointed out that the animal may have to contend not only with desiccation but with excessive hydration. The physiological consequences of hydration appear to be met by the flame-cell mechanism.

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The Behaviour of Mammalian Skin Epithelium under Strictly Anaerobic Conditions

BY

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With eight Text-figures

IN the course of some experiments on the nature of skin transplantation immunity, it became necessary to find out for how long (if at all) mammalian skin epithelium can survive in the total absence of atmospheric oxygen. The results of the investigation are summarized here in the hope that they may be useful to other students of tissue behaviour.

METHODS

Principle. Small squares of thinly sliced skin are removed from the body, placed in an adequate quantity of a 'physiological' fluid, incubated in an atmosphere of hydrogen, and then, after varying periods, returned by a grafting operation to the animal from which they originally came. The outgrowth of epithelium from such grafts constitutes a proof of their survival that is hardly subject to error (Medawar, 1944).

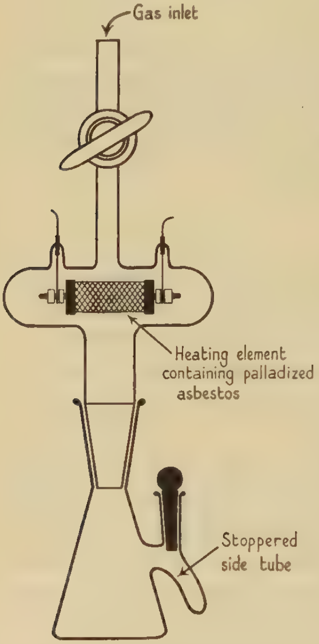
Preparation of the skin slices. Rabbits' skin has been used throughout. The skin of the chest or belly is shaved 4 days before the experiment, in order to toughen the otherwise very fragile cuticular layer and to thicken the epidermis. It is undesirable to use so-called 'pigmented' skin or skin of the spongy hyperaemic type that grows hairs very quickly. Skin of this active type may have an abnormally high metabolic requirement (cf. Peyton Rous, 1946).

On the day of operation, the rabbit is anaesthetized with ether and the shaved skin area washed for one minute with a cotton-wool swab soaked in a 1½ per cent. solution of cetyltrimethyl-ammoniumbromide ('CTAB', 'Cetavlon', a cationic soap: Barnes, 1942). The soap is removed with a fine jet of surgical spirit, and the skin surface at once dried with sterile swabs and very thinly coated with sterile vaseline applied with a glass rod. A central part of the area is divided into a grid of squares of side 3 mm. by very light scalpel incisions; the squares are then sliced off by using a 'Durham Duplex' razor blade firmly clamped in stout Spencer-Wells forceps, or a newly stropped straight-edge scalpel blade (e.g. Swann-Morton No. 11). After cutting, the grafts are placed raw side down in a Petri dish containing a circle of filter paper that has been damped with Ringer's solution.

Preparation of the incubation media. Serum is prepared from the skin donor by withdrawing 10–30 ml. blood from the median artery of the ear, previously washed, shaved, and rendered hyperaemic by rubbing with a little xylene. The blood is allowed to clot spontaneously and then briskly spun. The saline solutions that replace serum in some of the experiments described below are Krebs-Ringer-bicarbonate or Krebs-Ringer-phosphate, in each case containing a final concentration of 1 : 100,000 phenol red and 20 u./ml. streptomycin (see below). The 'iodoacetate' referred to below is prepared by dilution of an M/100 solution made up as follows: 0·465 g. monoiodoacetic acid is dissolved in about 200 ml. water containing 2·5 ml. 1 : 1000 phenol red. 2·50 ml. N/1 NaOH solution is run in slowly, and the volume made up to 250 ml. by the addition of further water.

Technique of incubation. The 'cultivation' of the skin squares is simply a matter of floating them raw side down, in pairs, on 5 ml. culture fluid in standard 50-ml. conical flasks with B-19 size ground-glass stoppers. In 'aerobic' control experiments the gas medium is simply air. It is desirable, but not absolutely necessary, that the flasks should be shaken from side to side during the run of the experiment by a slow tilting or rocking motion; and also desirable, but again not necessary, that the flasks should be incubated in a 38° C. water bath rather than in a bacteriological dry air incubator.

The technique for anaerobic incubation requires the fitting of a similar conical flask with a stoppered side tube (as in manometric apparatus) to the 'unit' illustrated by Text-fig. 1. The principle of anaerobic culture is to grow the tissues in an atmosphere of cylinder hydrogen from which the last traces of oxygen have been catalytically removed by a heating element wrapped around asbestos impregnated with metallic palladium. This technique was introduced in bacteriology by Laidlaw (1915), and the 'element' illustrated in Text-fig. 1 is the standard fitting supplied with McIntosh-Fildes's anaerobic culture jars. The side tube of the culture flask contains an oxygen indicator.



TEXT-FIG. 1. Anaerobic incubation apparatus.

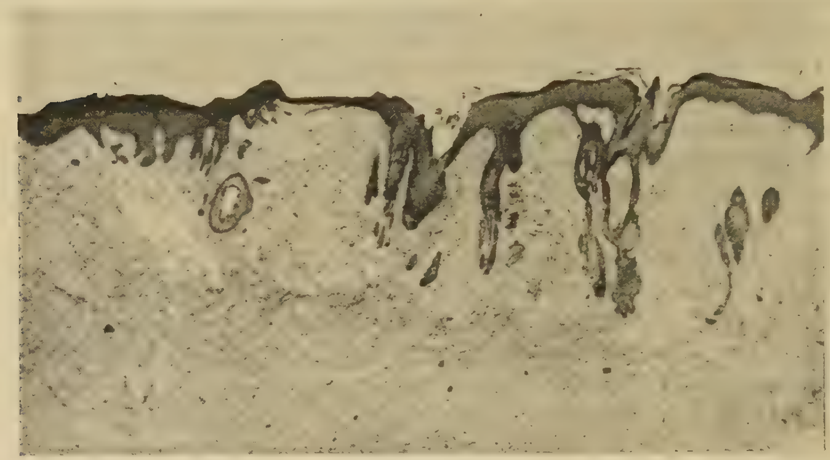
The following is a step-by-step account of a method that has given satisfactory results. Five ml. culture fluid are put into the culture flask, followed by the two skin squares. The side tube is then filled with about 1 ml. indicator solution freshly mixed from the following stock ingredients: 1 : 1000 methylene blue, 2 ml.; 1 per cent. glucose in water, 8 ml.; N/1 NaOH, 5–7 drops. (It might seem preferable to use an indicator with a more negative oxidation-reduction potential, such as neutral red reduced with sodium hyposulphite ('hydrosulphite', $Na_2S_2O_4$); but this system is difficult to work with, and since the indicator is meant to reveal leaks and not to measure O_2 tension, the methylene blue is perfectly satisfactory.)

The loaded culture flask is attached to the unit, and H_2 gas from a cylinder with a reduction valve and water trap is passed briskly through the apparatus for 2 minutes. The gas escapes through the side-tube stopper, left slightly open. The flow is then reduced to a trickle, the side-tube closed, and current passed through the terminals of the heating element, using 60-W. and 40-W. gas-filled light bulbs in parallel as resistances. Water is soon seen to condense on the glass housing the element. Half an hour's passage of current is more than sufficient; the top tap is closed, the current switched off, and the whole apparatus incubated at 38° C. The indicator becomes white after an hour or so of incubation. It will do so earlier if the NaOH concentration is raised, but this is unnecessary.

At the end of the experiment the skin slices are rinsed in Ringer's solution; one is grafted back to its donor, and the other kept for histological examination. When air is admitted, the heating element will be seen to 'steam' and the indicator to turn blue.

Note on asepsis. All the operations described above were done aseptically; but skin cannot be wholly freed from its 'resident flora', no matter how much care is taken, so there is always a danger that the experiment may be ruined by infection. Streptomycin at a final concentration of 20 u./ml. in the culture medium has been found to abolish this danger: it is not visibly harmful to skin epithelium at ten times this concentration, and has now been used for two years as a routine measure in all the writer's tissue-culture experiments with skin. (The sample used (1 mg. = 250 u.) was kindly supplied by Dr. E. S. Duthie, who suggested its use for tissue-culture purposes (cf. also Heilman, 1945). Streptomycin, discovered by S. A. Waksman (cf. Waksman, 1945) is an antibiotic base recovered from the soil fungus *Actinomyces* (*Streptomyces*) *griseus*: unlike the acidic penicillin, it is active against Gram-negative organisms.) It has proved convenient to prepare a solution containing 200 u./ml., sterilized by passage through a bacteria-retaining sintered glass filter, and to dilute it before use with nine times its volume of the culture medium.

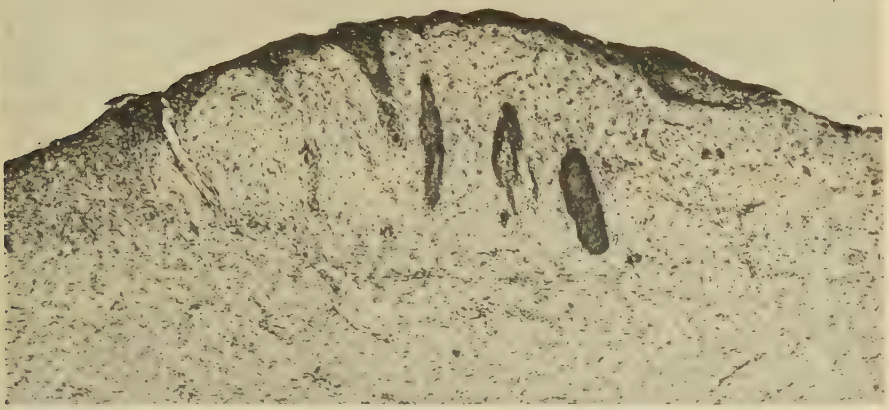
The test of survival. Tissue-culture itself offers one obvious method by means of which the viability of cells presumed alive or dead may be put to the test. For testing the viability of skin, however, transplantation technique is in every respect (and above all, in sensitivity) much superior. The skin squares are rinsed in Ringer's solution and, after aerobic cultivation only, their dermal surfaces are gently scraped to remove the epithelium that will have grown over and encysted them. The rabbit that provided the grafts is then shaved over the right side of the chest, and the skin so exposed rubbed with 5 per cent. aqueous 'Dettol' solution, which is allowed to dry on. (It is possible but not very desirable to use a rabbit other than that which provided the skin squares. As the grafts are small, and represent a fairly low 'dosage' of foreign homologous skin, they can be expected in the majority of cases to survive and proliferate during the 10-day test period without serious interference from the homograft immunity reaction (cf. Medawar, 1944, 1945).) A square of skin of 5 cm. side is then stripped off the sterilized area in the natural 'splitting' plane that lies between the panniculus carnosus beneath and the compact collagenous tissue of the dermis above. Every tag of skin that might house the base of a hair follicle must be removed—a very easy operation, unless the skin is unusually thick or the incision insufficiently radical. The skin grafts are blotted dry on sterile gauze and laid raw side down on the centre of the raw area, which is then thickly dusted with sterile sulphadiazine powder, covered with a widely overlapping square of vaseline-impregnated gauze ('tulle gras'), and a square of several thicknesses of dry gauze. The thorax is thereupon firmly wound, first with 1 yard of 3-in. bandage, and then with 18 in. of plaster-impregnated 4 in. bandage (e.g. 'Gypsona'). After the plaster has dried, the animal requires no further special attention until the inspection 10 days later.



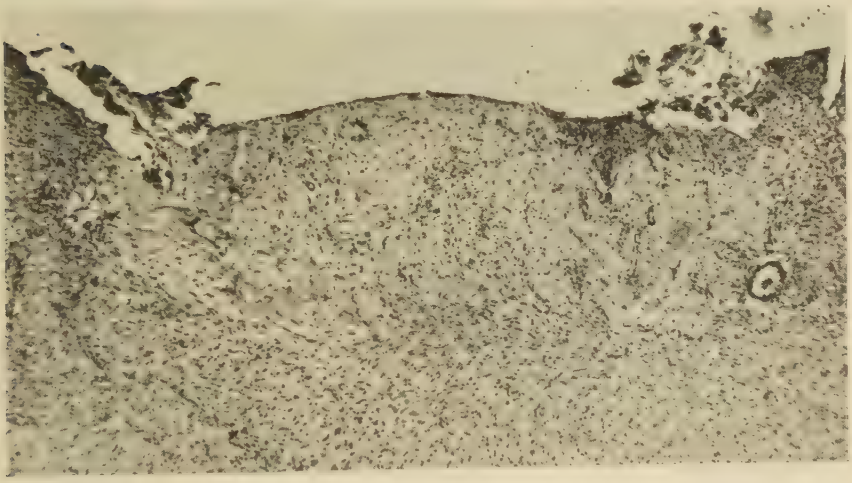
TEXT-FIG. 2. Part of a 10-day test graft showing full survival: note the outgrowth over the raw area of thick hyperplastic epithelium.

If the graft proves at this inspection to be surrounded by an annulus, 2–5 mm. wide, of thick and glistening white epidermal epithelium that has spread from it over the surrounding raw area, the result of the experiment may be recorded as 'survival' without further ado. In any other event the graft should be removed and examined by sections cut at four or five levels and stained with Ehrlich's haematoxylin and eosin. If only a trace of follicular epithelium remained alive in the original skin square, it may not have had time during the 10-day test period to do more than proliferate locally and force its way to that part of the graft surface that immediately overlies it. Naked-eye inspection alone hardly makes it possible to distinguish between this state of affairs and that in which the graft fails altogether to survive.

Text-figs. 2, 3, and 4 illustrate the histological appearances of test grafts showing full survival, partial survival, and no survival respectively. Each was removed 10 days after transplantation. The first two yield a positive result—survival—and this can hardly be subject to error. The third result—non-survival—must be subject to a small and consistent error: for if only a dozen or so cells remain alive in the incubated skin square, they may not be able to survive the further short period of ischaemia entailed by the grafting operation.



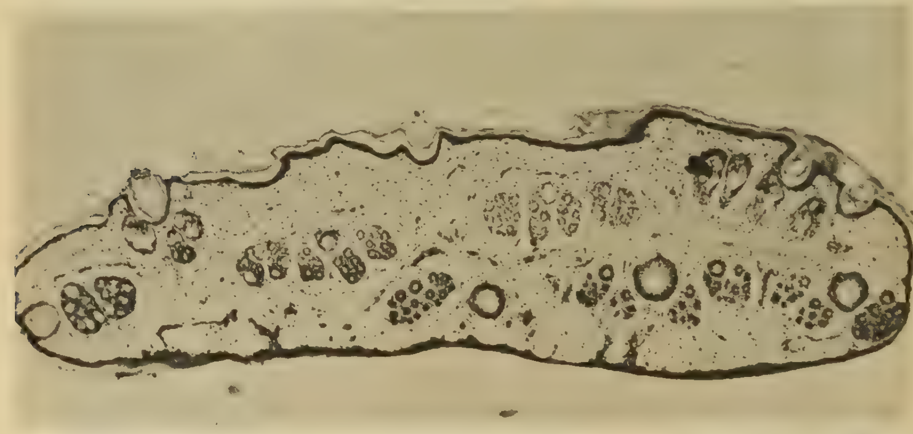
TEXT-FIG. 3. Ten-day test graft showing partial survival. Follicle epithelium has forced its way to the graft surface, but there is no outgrowth over the raw area.



TEXT-FIG. 4. Ten-day test graft showing no survival. The dermis of the graft is deeply embedded in the granulation tissue into which the surrounding raw area has become transformed. There is no surviving epithelium.

RESULTS

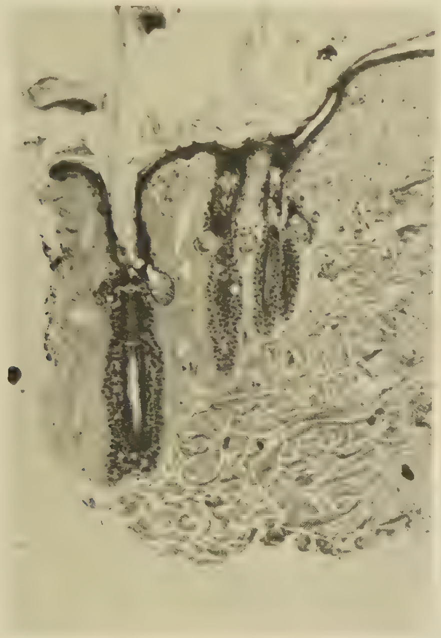
(A) *Behaviour of skin incubated aerobically in serum.* Skin squares incubated for 4 days in 4·5 ml. autologous serum+0·5 ml. streptomycin 200 u./ml. show survival and proliferation of all epidermal elements and a strong migratory overgrowth (epiboly) round the dermal pad that bounds the skin square below. In consequence, the skin square encysts itself in epithelium (Text-fig. 5). Growth is better in a stirred medium, and better still if blood corpuscles are added in physiological concentration to the serum to give an artificial non-clotting 'whole blood'. A full description of such skin cultures will be published elsewhere; the only conclusions relevant here are that cultures incubated aerobically in serum show full survival, migratory overgrowth, and cell division. (Migratory overgrowth is the equivalent of 'outgrowth' in conventional tissue-cultures.)



TEXT-FIG. 5. Skin square incubated aerobically in serum for 4 days. Migratory overgrowth of the epithelium round the dermal surface has brought about complete encystment.

(B) *Behaviour anaerobically.* Skin incubated anaerobically for 4 days in serum with streptomycin shows either full survival, as indicated by the grafting test, or something very little short of it. As incubation proceeds beyond the fourth day, the quantity of outgrowth from the test graft progressively falls off; until, by the eighth day, it is reduced to scraps of hyperplastic follicle epithelium, which may have migrated upwards to form a secondary coating of surface epithelium immediately above them (Text-fig. 3). Beyond 8 days of incubation, the test graft shows no survival (Text-fig. 4). However, the addition of extra glucose to the serum (0·5 per cent. above the original concentration) proved in each of two independent trials to prolong survival to the ninth day at least.

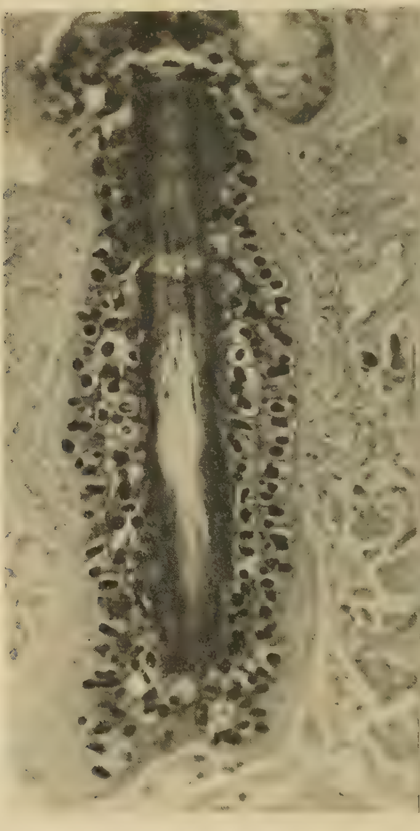
Histological analysis of the skin squares, after incubation but before test grafting, was made by fixing one of the two pieces normally housed in each vessel. Skin cultivated anaerobically shows neither cellular movement nor ell division: the edges of the skin square, instead of being rounded off by pithelial overgrowth, remain as sharp as they were when originally cut vith a scalpel blade (Text-fig. 6). A most determined search for mitotic igures in sections stained with Heidenhain's haematoxylin revealed none. There is not even any migration of follicle epithelium towards the skin urface: the hair bases remain firmly gripped in their epithelial jackets



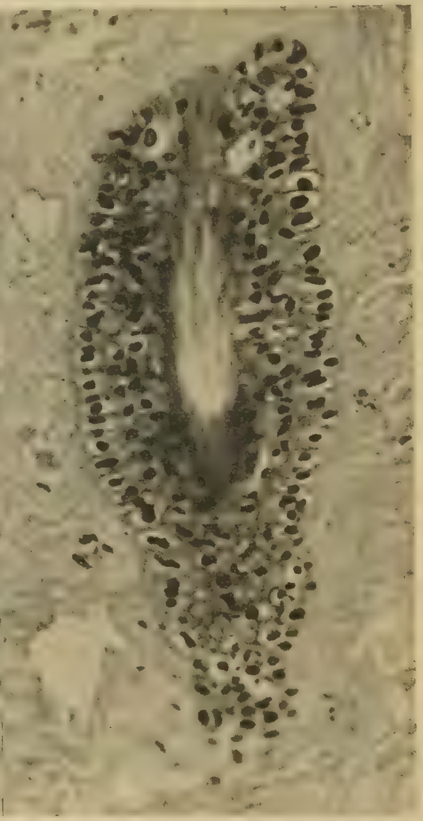
TEXT-FIG. 6. Contrast with Text-fig. 5: the edge of a skin slice incubated anaerobically in erum for 4 days. The slice ends off abruptly; there has been no migratory overgrowth, nor ny upward migration of follicle epithelium. The marginal cells of the surface epithelium ave undergone maceration, but the follicle epithelium is in a surviving state.

Text-fig. 6) and the follicles show none of that flare-like enlargement that is ndicative of upward migration.

The majority of the incubated skin squares were fixed in $HgCl_2$-formal-ehyde; dehydrated and cleared in an ethyl alcohol series, cedarwood oil, and groin; embedded in paraffin wax; cut at 8μ thickness; and stained with hrlich's haematoxylin and eosin. After this treatment the cells of the deep ollicle epithelium present a characteristic and uniform appearance (Text-gs. 7 and 8). The cytoplasm is weakly basiphilic and diffuse or cloudy in eneral staining reaction. The nuclei, instead of being large and juicy in ppearance, give the impression of being withdrawn from the cytoplasm, are regular if not frankly crenated in outline, and stain rather densely—though ot in the fashion described by pathologists as 'pyknotic'. The surface pithelium is much more variable. After short periods of incubation the cells are typically in the condition just described; but after longer periods they may give the characteristic appearance of aseptic necrosis. The cells may have separated from each other, or the reaction of the cytoplasm may be acidophilic and the nuclei represented by ghost outlines alone. Cells of this appearance are presumably dead; but those described beforehand, though an histologist might well call them highly abnormal and degenerate, are in fact 'alive' i the sense defined by the grafting test. A cytochemical study of the cells outside the scope of the present inquiry, might be very revealing.



TEXT-FIG. 7.



TEXT-FIG. 8.

TEXT-FIG. 7. The follicle epithelium illustrated by Text-fig. 6: higher power. For description, see text. (The sections illustrated in Text-figs. 7 and 8 have been photographe in such a way as to bring the nuclei into the highest possible contrast; their frankly pyknoti appearance is thus largely a photographic artifact.)

TEXT-FIG. 8. Cf. Text-fig. 7: another island of deep follicle epithelium; from a graft incubated anaerobically for 6 days in serum.

It appears, then, that cellular survival is sharply dissociable from cel division and cell movement; and that the presence of molecular oxygen i a necessary condition for division and movement, but not for mere survival

So far as can be judged by histological examination alone, dermal mesen chyme cells (fibroblasts, histiocytes, and vascular endothelium) may survive anaerobically for 4 or 5 days. They are not as resistant to anaerobiosis as is skin epithelium, and after 6 or 7 days are reduced to pyknotic or fragmented scraps of nuclear material uninvested by cytoplasm.

(C) *Behaviour in physiological salt solutions.* All the experiments to be described under headings (C) and (D) make use of a 4-day incubation period; a period chosen because after 4 days of anaerobic incubation in serum survival is still complete or falls little short of it.

Skin slices will survive aerobic incubation for 4 days in Krebs-Ringer-bicarbonate or Krebs-Ringer-phosphate, in either case with *or without* the addition of glucose (0·5 per cent. final concentration): they will *not* survive 4 days' anaerobic incubation, without *or with* glucose in the same concentration. (The phosphate-buffered Ringer is the medium of choice for anaerobic experiments of the type described in this paper. The reaction of a bicarbonate-buffered medium becomes progressively more alkaline during the course of the experiment, presumably because of the escape of CO_2 into the hydrogen gas phase.)

(D) *The iodoacetate-sensitivity of skin slices.* Skin incubated aerobically *in vitro* has proved to be very sensitive to iodoacetate. An attempt was first made to determine the threshold concentration of iodoacetate that *just* permits the survival of skin after 4 days' aerobic incubation in a medium containing autologous serum 4·0 ml., streptomycin (200 u./ml.) 0·5 ml., and iodoacetate solution 0·5 ml. The accompanying table sets out the results of 7 independent experiments: '1' stands for 'survival' and '0' for non-survival in the test grafts.

	1	*2*	*3*	*4*	*5*	*6*	*7*
M:7,500 . . .				0	1	0	0
M:10,000 . . .	1	1	0	0	1	0	0
M:12,500 . . .				0	1	0	1

Five independent preliminary trials at a concentration of M : 4,000 had given uniformly negative results. The results are evidently somewhat variable, though they put the order of magnitude of the minimal lethal concentration at M : 10,000. Part of the variation may be attributable to the presence in serum of a variable quantity of a glycolysis-inhibiting factor (cf. Elliot and Henry, 1946).

More consistent results were obtained from four additional independent tests in which serum was replaced by Krebs-Ringer-phosphate containing 0·5 per cent. glucose, and a slightly wider spacing of iodoacetate dilutions was used:

	8	*9*	*10*	*11*
M:5,000 . . .	0	0	0	0
M:10,000 . . .	0	0	0	0
M:15,000 . . .	1	0	1	1

The three 'positives' recorded under M : 15,000 were not, however, of equal value: in one of the three positive cases survival was 'partial' only; in the other two, considerable but probably not complete. Since rabbit skin varies a good deal in 'activity', i.e. in its intrinsic rate of cell division on removal, it is doubtful if these results justify a more definite conclusion than that the order of magnitude of the minimal lethal concentration of iodoacetate, after 4 days' exposure, is of the order of 10^{-4} molar.

DISCUSSION

The fact that epidermal movement and cell division is brought to a standstill under anaerobic conditions is not unexpected (cf. Needham, 1942; Kitching and Moser, 1940; Ormsbee and Fisher, 1944). It is surprising that the power merely to survive 'vegetatively' at body temperature is so sharply dissociable from migratory and karyokinetic activity; but the experimental results show quite clearly that skin epithelium may survive anaerobically in serum for periods upwards of a week at body temperature. *Paramecium*, so Kitching (1939) found, can live anaerobically only for a matter of hours—though he quotes earlier estimates ranging from a few seconds to a few months!

Skin epithelium is a tissue that depends for its blood-supply upon the capillary circulation in the upper reaches of the dermis. Presumably, therefore, it has to submit to low oxygen tensions as a matter of course, and relies to a large extent upon a glycolytic mechanism. Berenblum, Chain, and Heatley (1940) and Dickens and Weil-Malherbe (1943) find that skin epithelium displays aerobic as well as anaerobic glycolysis; Dixon and Needham (1946), by contrast, find that the abdominal skin of 4-day old rats shows no aerobic glycolysis but metabolizes after the fashion of skeletal muscle.

If it is correct to assume that iodoacetate in a concentration of the order of 10^{-4} molar is a specific inhibitor of glycolysis, then these two definite conclusions may be drawn from the experiments described in the text: the survival of skin epithelium depends upon the integrity of its glycolytic mechanism; but cellular movement and division depend upon respiratory activity in the narrow sense. It may be added, though at present only on the basis of rough observations, that the rate of cell division in skin epithelium varies directly with O_2 tension over a fairly wide range. The behaviour of skin cultivated *in vitro* (to be described elsewhere), and of the cornea *in vivo*, shows that epidermal epithelium does not need much oxygen in order to grow and move. It does, however, need some. It is possible that cartilage cells behave in like manner (Bywaters, 1937).

SUMMARY

1. The viability of skin epithelium subjected to various experimental treatments may be tested by transplantation technique.

2. Skin can survive for upwards of a week in serum at body temperature in the total absence of atmospheric or dissolved oxygen.

3. Neither cellular movement nor cell division can take place in skin epithelium under anaerobic conditions.

4. The survival of skin epithelium depends upon the integrity of a mechanism, presumably glycolysis, that is put out of action by iodoacetate at a concentration of the order of 10^{-4} molar.

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Further Observations on the Glomerular Root of the Vertebrate Kidney

BY

J. F. A. McMANUS

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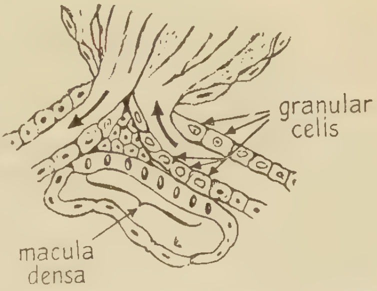
With five Text-figures

INTRODUCTION

IN the kidneys of a wide variety of animals the second convoluted tubule is applied to the root of the glomerulus of the same nephron (Text-fig. 1). Here the tubular cells are aggregated to form the *macula densa*. In these cells he Golgi element lies on the side of the nucleus opposite to that on which it ies in the rest of the tubule (McManus, 1943, 1944). The tubule is set into he angle between the afferent and efferent rterioles of the glomerulus (Text-fig. 2A). Rotation of the plane of section through 90° produces the appearance shown in Text-ig. 2B. The arteriolar cells in this region re known in a variety of species to possess granules in their cytoplasm.

These relationships become established t a very early stage in embryonic development. In the human foetus, and in every other embryo examined from fishes upwards, a segment of tubule applied to the glomerular root can be recognized as soon as there is a glomerulus supplied by two vessels destined to become the rterioles.

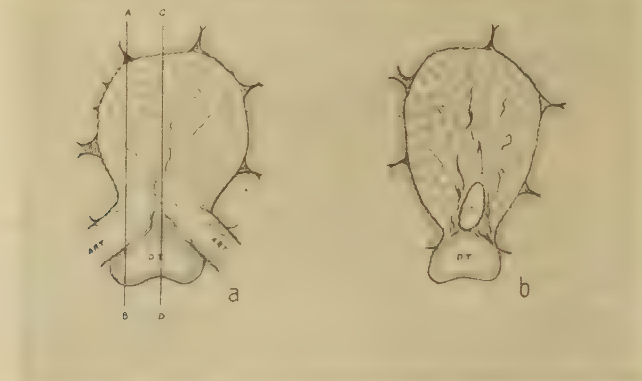


TEXT-FIG. 1. Glomerular complex. Diagram showing relationships at root of glomerulus. From *Lancet*, 1942.

Since the last review of the structure of the glomerular root was written McManus, 1942), not a great deal has been published about the problem of he role of the components of the glomerular or juxta-glomerular complex. Goormaghtigh (1944) has continued to present evidence suggesting an ndocrine activity for the granular cells, and has recently (1945) described he hyperplasia of these cells in the so-called 'crush' kidney, that is, the human idney after severe wounds in other parts of the body, especially of the limbs. The present communication describes further findings concerning the cyto-ogy and histology of the glomerular root.

Reticulin Patterns at the Glomerular Root

The distribution of reticulin has been studied by various modifications of Robb-Smith's (1937) method of floating paraffin sections on the solutions used. Foot's technique (1924) was used, as Robb-Smith recommends, as well as Divry's (1932) modification of Hortega's silver carbonate. The use of thinner sections made possible by this 'floating' routine has permitted the recognition of features that are obscured in thicker sections, and especially in thick frozen sections.



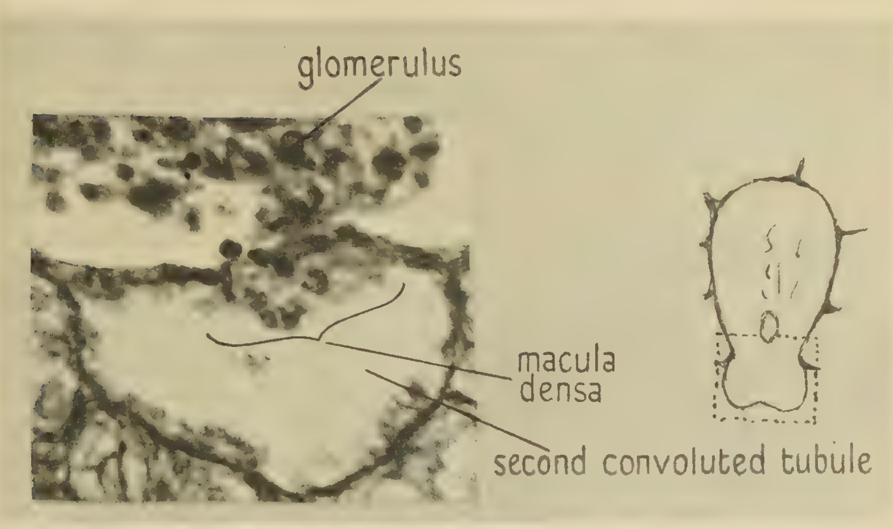
Text-fig. 2. Diagrams of reticulin about glomerulus. Text-fig. 2B is at plane AB of Text-fig. 2A.

A complete basement membrane, on which the epithelial cells rest, surrounds each tubule and inter-tubular fibrils connect these membrane units with the vascular tree of the kidney. Further subdivisions of the reticulin fibrils have been described by Tello (quoted by Ramon-y-Cajal, 1933), and the development and age-changes have been discussed by Schwab (1939). These authors do not describe the arrangement of the reticulin at the glomerular root.

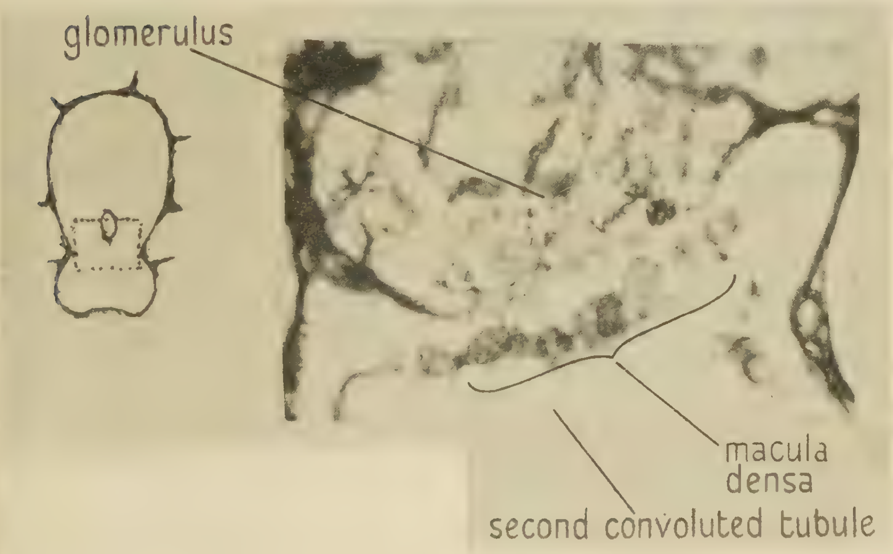
I find that there is always a gap in the reticulin at the glomerular root. Reticulin is totally absent between the tubule and vessels in the mouse, cat (Text-fig. 3), and rabbit, and there are multiple gaps in adult man (Text-fig. 4). The condition in the child closely resembles that in the mouse, cat, and rabbit. Thus the cytoplasm of the cells of the macula densa is only separated from that of the cells of the arterioles by the cell-membranes. The absence of any basement membrane at the glomerular root would facilitate exchange between these cells of the tubule and the arteriolar cells, which in this situation possess the specific granules.

The Composition of the Granules in the Cells of the Arterioles

Ruyter in 1925 described granules in the juxta-glomerular cells of the afferent arteriole in the mouse. Oberling saw them in man, Okkels in the frog, and

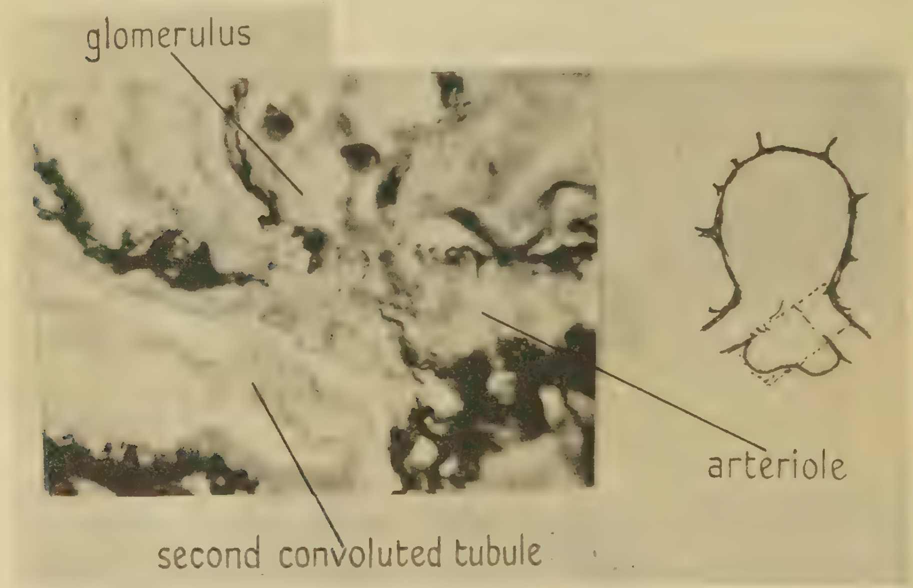


Text-fig. 3. Part of a section of the kidney of a kitten. (Stained for reticulin.)



Text-fig. 4. Part of a section of a normal human kidney. (Stained for reticulin.)

Goormaghtigh in the cat and rabbit (references in McManus, 1942). They have been studied by what are essentially mitochondrial methods, which are notoriously capricious. Although a few workers have subsequently recognized and studied them (Kaufmann, Donihue, and Candon), the methods for their demonstration have been unreliable, and there has consequently been delay in the general investigation of them.



TEXT-FIG. 5. Part of a section of a human kidney affected by malignant hypertension. Note increase in reticulin (? arteriosclerosis), but persistence of gap. (Stained for reticulin.)

The granules are perfectly shown in the mouse by the use of a cobalt-calcium-formalin fixative (McManus, 1946), followed by either sudan black or Masson's trichrome stain. The mitochondria are well preserved and the method seems suitable for a new attack on the kidney. In the rabbit, unlike the mouse, postchroming is necessary and it is advisable to dehydrate in acetone. The same colouring methods are suitable. The human kidneys I have been able to study were not normal but 'crush' cases, in which the granular cells of the arterioles seem to be very numerous. As with the rabbit, postchroming is necessary but alcohol can be used for dehydration. Once again the granules can be shown with sudan black or Masson's trichrome stain. The similarity of staining reactions suggests that the granules of the juxtaglomerular arteriolar cells of the various species are of similar chemical composition. Colouring by sudan black after fixation in the cobalt fixative and imbedding in paraffin shows that the granules consist (at least in part) of masked lipoid (perhaps lipine).

THE RELATION OF THE GRANULAR CELLS TO THE RENAL TUBULE

The distribution of masked lipoid in the kidney, especially in the mouse, but also in the rabbit and man, as shown by the cobalt-sudan-black technique and by later variants of the same method, presents features that would interest students of the kidney. The clearness with which the granules are shown is striking. The part of the afferent arteriole nearest the glomerulus seems to be composed entirely of these granular cells in the case of the mouse. Hardly less striking is the complete absence of masked lipoid in the macula densa. There is an equally strong contrast in this respect between the macula cells and the adjacent cells of the same tubule, which are rich in masked lipoid.

The facts suggest that something passes between the lumen of the tubule and the cells of the arteriole, and that this movement is accompanied by the movement of lipoids, which aggregate in the arteriolar cells. The direction of flow is suggested by the reversal of the Golgi element in the cells of the macula densa, for in this region—and in this region alone—the Golgi element lies on the side of the nucleus directed towards the base of the cell, that is, towards the side on which there is contact with the granular cells of the arteriole.

In a limited series of cases of chronic nephritis which have already been mentioned (in McManus, 1942), the tubules were not uncommonly found to be separated from the arterioles. I have now somewhat extended the investigation of these and similar cases, especially by studying the distribution of reticulin. The gap in the reticulin at the glomerular root appears often to be obliterated in some cases of chronic glomerulonephritis, but not in the cases of arteriosclerosis or malignant hypertension (Text-fig. 5) which were studied.

SUMMARY

There is a gap in the reticulin at the glomerular root of the mammalian kidney. This gap leaves the macular segment of the second convoluted tubule in intimate contact with the arteriolar cells, which in this region contain granules consisting of or containing masked lipoid. Methods are given for the demonstration of these granules in the mouse, rabbit, and man.

The cells of the macula densa of the mouse are poor in lipoid, and there is thus a striking contrast both with the adjoining cells of the same tubule and with the adjacent arteriolar cells.

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The Action of Diethylstilboestrol on the Prostatic Epithelium of the Mouse

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With one Plate and and Text-figure

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1. INTRODUCTION

THE gross effects of oestrogens on the male reproductive system in rodents have been described by numerous authors as involving testicular degeneration, and hypertrophy of the accessory glands and ducts. While the seminal vesicles of rats undergo fibrosis and growth of smooth muscle without any epithelial changes, both the prostate and seminal vesicles in mice show epithelial hyperplasia, followed by metaplasia and keratinization after prolonged treatment. The initial stages of this oestrogenic stimulation of the prostatic epithelium in mice have not been investigated with cytological techniques. Moore, Price, and Gallagher (1930) described characteristic cellular changes in the prostatic epithelium of rats in response to castration and to injection of preparations containing androgens. Consistent morphological variations in the Golgi apparatus of the epithelial cells were found to accompany epithelial regression or hypertrophy. Thus the presence of androgen in a preparation could be tested by injection followed by cytological examination of the prostatic epithelium.

Experiments were undertaken to determine when structural changes may first be detected in the epithelial cells of the various lobes of the mouse prostate following oestrogen administration, and to follow the recovery stages after the treatment was discontinued. The cytological response of the epithelial cells was investigated in order to determine whether male uncastrated mice might be used in testing for the activity of oestrogen.

2. Material and Methods

Young male mice belonging to an inbred strain (R III) were selected for these experiments. A total of 32 mice, aged 4–5 months, received 10 mg. pellets of the synthetic oestrogen diethylstilboestrol by subcutaneous implantation, according to the technique of Deanesly and Parkes (1937). A number of mice were sacrificed at intervals from 4 to 20 days after treatment. Ten untreated mice were used as control material. In order to study the cytology of the recovery phases of the prostatic epithelium following oestrogen administration the pellets were removed after 20 days' implantation. A number of mice were then killed at periods ranging from 10 to 20 days after oestrogen treatment had ceased.

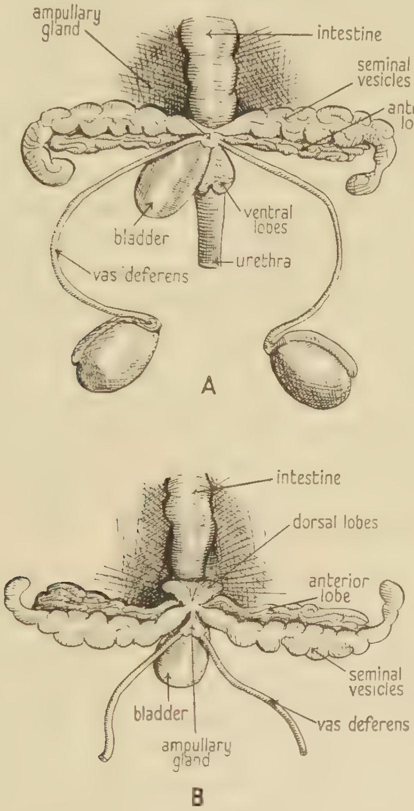
The anterior, dorsal, and ventral prostatic glands were removed under a binocular dissecting microscope for separate fixation. Preparations of the Golgi apparatus were obtained by treating the tissues by Kolatchev's method as modified by Nassonov. All sections were subsequently stained with Orange G in clove oil. Histological preparations were made from material fixed in Dubosc-Brasil's modification of Bouin's solution and stained by Masson's light green-eosin technique.

3. The Structure of the Normal Prostatic Gland in R III Mice

The nomenclature of the prostatic lobes in rodents has been a subject of much confusion (Rauther, 1903; Disselhorst, 1904; Walker, 1910; Burrows, 1934; Korenchevsky and Dennison, 1935). The several glands, generally referred to as the lobes of the prostate in the mouse, have been described by Deanesly and Parkes (1933) as consisting of paired anterior, ventral, and dorsal lobes, together with a small median gland (see Text-fig. 1A). The last, more frequently termed the ampullary lobe (Fekete, 1941), is a tubular gland lying adjacent to the ampulla in close connexion with the ductus deferens. It opens into the anterior vestibular portion of the ampulla. Examination of this structure in a number of mice belonging to pure line strains showed much variation in size within different strains. In R III mice only a vestigial gland is present.

The paired anterior lobes, called the coagulating gland by some authors (Burrows, 1934; Fekete, 1941), are imbedded in the peritoneal sheath of the seminal vesicles and open into the dorsal wall of the prostatic urethra. The lining epithelium consists of tall columnar cells arranged in characteristic folds. Moore (1939) and others refer to these anterior lobes as the true prostate.

The paired dorsal prostates, which are very much narrower than the anterior lobes, are nevertheless similar in structure and are attached to the dorsal wall of the urethra (see Text-fig. 1B). The columnar epithelium is less folded than in the anterior lobes. The ventral prostate also consists of bilaterally symmetrical lobes situated on the ventral wall of the urethra at the base of the bladder, and the tubules composing this portion of the prostate differ from those of the anterior and dorsal glands in that they are lined by a low columnar epithelium which is unfolded. The ventral lobes possess several ducts which open into the urethra on its ventral surface.



TEXT-FIG 1. Drawings showing ventral and dorsal views of the lobes forming the prostate gland, seminal vesicles, and bladder in a mouse of the R III strain.

4. CYTOLOGY OF THE UNTREATED PROSTATIC EPITHELIUM

Characteristic differences exist between the morphology of the Golgi apparatus in the normal untreated epithelia of the anterior, ventral, and dorsal prostatic lobes. These differences are independent of the normal structural variations occurring in the glandular epithelium during secretory activity (see Pl. 1, figs. 1, 3, 5). Before any significance can be attached to the extensive changes in the Golgi material found after short periods of oestrogenic treatment (see Pl. 1, figs. 2, 4, 6), it is essential to record the cytological appearances of the normal epithelium.

Anterior Prostates. The cells of the columnar epithelium possess a slightly granular cytoplasm and almost spherical nuclei, with the Golgi apparatus forming a condensed flattened network restricted to the apical region of the cytoplasm. The apparatus in the majority of cells forms an elongated body, orientated transversely to the axis of the cell and situated in close contact with the nuclear membrane (see Pl. 1, fig. 5). Where the mucous membrane is thrown into tall longitudinal folds, projecting far into the lumen, the Golgi substance then becomes compressed and distorted, often losing the appearance of a network and assuming a more compact rounded shape.

Dorsal prostates. The cells of the dorsal prostatic epithelium resemble those of the anterior lobes. The general cytoplasm is, however, more granular and the spherical nuclei stain more deeply. The Golgi apparatus, situated in the apical cytoplasm in close contact with the nucleus, forms a network which is less compact than that found in the anterior lobe cells (see Pl. 1, fig. 3). In the proximal region close to the basement membrane the cells contain secretion droplets which are stained lightly by osmium tetroxide.

Ventral prostates. The low columnar epithelium of the ventral lobe is relatively free from folds. The individual cells are elongated with their oval nuclei confined to the basal region of the cytoplasm (see Pl. 1, fig. 1). The Golgi apparatus forms a characteristic loose network in the apical cytoplasm of these cells and consists of twisted strands, all of which tend to be orientated longitudinally to the axis of the cell (see Pl. 1, fig. 1). The morphology and location of the Golgi material in the epithelial cells of the ventral prostate are therefore quite different as compared with the structure of the apparatus in the anterior and dorsal prostatic epithelia (see Pl. 1, figs. 3, 5).

5. RESPONSE OF THE PROSTATIC EPITHELIUM TO EIGHT DAYS' TREATMENT WITH OESTROGEN

Oestrogenic stimulation does not induce uniform changes throughout all the prostatic lobes. After 8 days' treatment the cytological appearances of the epithelia clearly demonstrate that differences in the degree of sensitivity exist between the three glands (see Pl. 1, figs. 2, 4, 6). There is clear indication that the epithelium of the anterior lobe is more sensitive to stilboestrol than either the epithelium of the dorsal or ventral lobes. The cytological changes which are typical after 8 days' treatment will be briefly described.

Anterior prostatic epithelium. On the eighth day a slight hypertrophy of the whole epithelium, involving an extensive fragmentation and dispersal of the Golgi apparatus throughout the cells, is a consistent feature of this period of treatment (see Pl. 1, fig. 6). The compact Golgi network of the untreated anterior glandular epithelium has entirely disappeared and the enlarged epithelial cells have become filled with fragments of hypertrophied Golgi substance. In most cells there is an aggregation of Golgi material in the apical cytoplasm in the vicinity of the nucleus, and the latter may be distorted. A dispersed Golgi apparatus occupies the entire cytoplasm (see Pl. 1, fig. 6).

Dorsal prostatic epithelium. The cellular changes in the dorsal epithelium are less extensive than those which occur in the anterior epithelium after the same period of treatment (see Pl. 1, figs. 4 and 6). There is less epithelial hypertrophy and the accompanying changes in the Golgi apparatus are less pronounced. The Golgi network undergoes only a slight fragmentation and occasionally an intact but enlarged Golgi reticulum may be observed in the apical cytoplasm in close relation with the nuclear membrane (see Pl. 1, fig. 4). The characteristic transverse polarity of the apparatus to the axis of the cell, so typical of the epithelium previous to treatment, is lost owing to fragmentation.

Ventral prostatic epithelium. The response of the Golgi apparatus to oestrogen in the ventral epithelium differs markedly from that in the epithelium comprising the dorsal and anterior prostates. After 8 days' treatment a definite but remarkably uniform hypertrophy of the apparatus occurs throughout the whole of the ventral prostatic epithelium. This is not accompanied by any enlargement of the epithelial cells nor by fragmentation or dispersal of the Golgi material. The compact apparatus is localized in the apical cytoplasm, and it still maintains its longitudinal polarity to the cell axis (see Pl. 1, fig. 2).

The difference in response of the Golgi apparatus in the epithelium of the dorsal, anterior, and ventral prostates to treatment can best be appreciated by an examination of Plate 1. The slight reaction of the Golgi material in the ventral epithelium suggests that it is less sensitive to oestrogen than either the anterior or dorsal epithelium.

6. RESPONSE OF THE PROSTATIC EPITHELIUM TO TWENTY DAYS' TREATMENT WITH OESTROGEN

The anterior prostatic epithelium on the twentieth day of treatment has undergone a conspicuous metaplasia. This phenomenon can be macroscopically observed upon laparotomy. At this period the paired anterior glands have become considerably enlarged and the seminal vesicles, which are embedded in the same peritoneal sheath as the anterior lobes, have atrophied. The hypertrophy of the anterior gland is partly due to distension of alveoli by keratinized epithelial debris and accumulation of polymorphonuclear leucocytes. The single layer of cuboidal epithelial cells, still present on the eighth day of treatment, is now replaced by a greatly hypertrophied stratified epithelium. Many of the alveoli are lined by an epithelium 10–15 cells in thickness.

It has been stated by Lacassagne (1933) and Burrows (1934) that the epithelial response of the anterior prostate in rodents to oestrogen provides evidence in support of the contention that these glands are derivatives of the Mullerian ducts. A similar process of rapid cornification of the vaginal epithelium in rodents occurs after short periods of treatment with oestrogens. During keratinization the Golgi bodies in the hypertrophied epithelial cells have undergone changes involving their morphology and distribution. The Golgi material is now present in the form of fine granules irregularly scattered throughout the cytoplasm. Occasionally cytoplasmic structures which stain more deeply than the Golgi material are seen lying adjacent to the enlarged nuclei. These structures, according to Ludford (1924), are nuclear extrusions.

giving rise to keratohyalin granules, and should therefore not be confused with Golgi substance. In most of the greatly enlarged keratinizing epithelial cells the scattered Golgi bodies stain only very faintly until they finally cease to be demonstrated by the Kolatchev technique. Ludford (1925) reports a similar phenomenon occurring in the keratinizing epithelial cells in tar tumours of rodents.

The cytological changes which have taken place within the same period in both the dorsal and ventral prostatic epithelium are less pronounced. The epithelium has undergone little cellular change and no keratinization occurs in these glands, a development entirely confined to the anterior prostate. There is little evidence of cellular proliferation at this stage of treatment, but in the dorsal glands some of the alveoli are now lined by two or three layers of cells. Apart from the increased fragmentation and subsequent dispersal throughout the cytoplasm of the Golgi material, the general cytological appearance of the epithelium is similar to that already described after 8 days' treatment. No desquamated cell debris is seen within the alveoli but numerous leucocytes are present. Proliferation of the ventral glandular epithelium is absent at this stage of treatment. The Golgi apparatus, which is now fragmented, still remains localized in the apical cytoplasm of the epithelial cells, close to the nuclear membrane.

7. THE RECOVERY PHASES OF THE PROSTATIC EPITHELIUM FOLLOWING CESSATION OF OESTROGENIC TREATMENT

The keratinized anterior prostatic epithelial cells return to the unstimulated condition 20 days after the removal of the oestrogen pellets. On the twentieth day the keratinized epithelium has disappeared and is replaced by a single layer of cuboidal cells, most of which appear to have resumed their normal secretory functions. Some of the alveoli still remain slightly distended with cell debris and a few epithelial cells may be enlarged owing to the accumulation of secretory products. In the majority of cells the Golgi substance has reformed into a compact but slightly larger network than that seen before treatment. Where the Golgi apparatus has not reformed itself into a network it still remains condensed and localized in the apical cytoplasm. It seems likely that normal secretory activity has not yet occurred in these cells, as it has been shown that oestrogens inhibit the secretory function of the prostate gland (Allen, 1939). The epithelium of the dorsal and ventral glands returns to a normal state more rapidly in accordance with their smaller response to oestrogenic stimulation.

8. DISCUSSION

The present experiments have shown that it is possible to correlate variations in the form and distribution of the Golgi material in the prostatic epithelial cells of the mouse with different periods of treatment with oestrogens, and that the normal morphology of the Golgi apparatus is restored in these cells when treatment is discontinued. It is evident that oestrogenic stimulation does not cause permanent alteration in the structure of the prostatic epithelium after relatively short periods of treatment, even when keratinization has been induced. The most extensive cytological changes following oestrogen treatment occur in the epithelium of the anterior lobes.

Amongst the more recent work published on the cytological effects of the sex hormones upon cells it should be noted that Moore, Price, and Gallagher (1930) report that the state of the Golgi apparatus in the rat prostatic epithelium has proved to be a reliable indicator for testing preparations for androgenic action. Vazquez-Lopez (1940) has shown that over-stimulation with oestrogen causes an extensive hypertrophy of the Golgi substance in the cells of the islets of Langerhans in the mouse pancreas. Wolf and Brown (1942) have recently described the reactions of the Golgi apparatus in the alpha and beta cells of the pars anterior of the pituitary body following treatment with diethylstilboestrol. In the uterine epithelium of the untreated rat during the oestrus cycle, it has been shown that normal oestrogenic activity is sufficient to produce characteristic cytological changes which are correlated with different phases of the cycle (Horning, 1943).

Before the introduction of the vaginal smear assay for oestrogen by Allen and Doisy (1923), it was generally held that any structural or functional change produced by ovariectomy, which could be restored to normal by injection of ovarian extracts, might be used for the assay of female sex hormone. The microscopic examination of the vaginal smear is regarded as a reliable indicator of the oestrous condition of intact female animals. Rodents, either previously spayed or immature, are employed for the testing of substances having oestrogenic action. This method requires a careful standardization of age and castration methods and has been criticized since it has been found that certain carcinogenic substances (Wright, 1936) are capable of producing the same effects as oestrogens in females. Hechter, Lev, and Soskin (1940) have reported that the alkaloid yohimbine also induces oestrus in rodents. Horning (1943) has suggested that the characteristic changes in the Golgi substance of the uterine epithelium in non-castrated mice, following 8 hours' treatment with diethylstilboestrol, might be used as reliable indicators of the degree of oestrogenic stimulation.

The effects of oestrogens upon male animals have been reviewed by Allen (1939). Golding and Ramirez (1928) were among the first to describe the specific action of oestrogen on immature male rats. They found that this treatment prevented the growth of the testes and their descent into the scrotum. Two weeks after oestrogen administration was discontinued, the treated animals had completely recovered from gonadal inhibition. The production of scrotal hernias in rodents, following relatively short periods of oestrogen treatment, has been reported by several workers (Lacassagne, 1933; Burrows and Kennaway, 1934; Cramer and Horning, 1938). Lacassagne (1936) has further described changes in the epithelium of the bladder in mice following similar treatment, and Gardner (1937) found a striking hypertrophy of the interstitial cells of the testis in response to oestrogens in certain inbred strains of mice. According to Burrows (1935) oestrogens cause the connective tissue of the ampullary region of the vas deferens in mice to undergo a marked myxomatous transformation after treatment with relatively large doses. He also reported (1937) that hyperplasia of the ducts of the bulbo-urethral glands occurred after similar treatment.

The present experiments have indicated that it is possible to use male animals, uncastrated and of any age, to test for oestrogenic activity. The cytological reaction in the epithelium of the anterior lobe of the prostate in particular is sufficiently characteristic and precise to serve as an indicator of oestrogen action, being clearly distinguishable from that induced in the same epithelium by the action of androgen. We now have, therefore, cytological criteria with which to assess the activity of oestrogens in the male and female rodent, as well as those originally described by Moore, Price, and Gallagher (1930) for androgens in the male. These methods will prove useful in supplementing the routine methods for the assay of sex hormones and may be employed in the investigation of the varying degrees of reaction to these hormones shown by the many types of epithelial cells and stromal tissues in the reproductive organs.

9. Summary

1. The cellular changes in the prostatic epithelium of an inbred strain of mice (R III), following short periods of treatment with large doses of oestrogen, are described.

2. The Golgi apparatus in the cells of the prostatic epithelium affords a precise indication of the degree of stimulation induced by oestrogenic substances.

3. Specific morphological changes in the Golgi substance occur in the epithelium of the anterior lobe of the prostate after 8 days' treatment. Similar changes, following longer periods of treatment, also occur in the dorsal and ventral lobes.

4. Withdrawal of oestrogenic stimulation after 20 days' treatment with relatively large doses is followed by a return of the prostatic epithelium and the Golgi apparatus to their normal condition in 21 days.

EXPLANATION OF PLATE I

Photomicrographs of the prostatic epithelium of treated and untreated R III mice. All material was impregnated by Nassonov's modification of Kolatchev's method, and subsequently stained with Orange G in clove oil. Magnifications, × 360.

Fig. 1. The prostatic epithelium of the ventral lobe of a control mouse. The columnar epithelium is relatively unfolded, and the Golgi apparatus forms a characteristic loose network in the apical cytoplasm. The Golgi material consists of twisted strands all of which tend to orientate longitudinally to the cell-axis.

Fig. 2. Ventral prostatic epithelium after 8 days' treatment with diethylstilboestrol, showing changes in the Golgi apparatus. It is hypertrophied but retains its compactness and normal polarity.

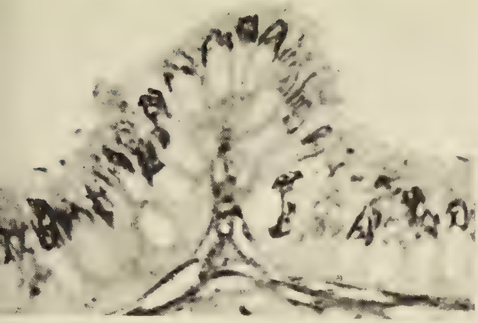


FIG. 1

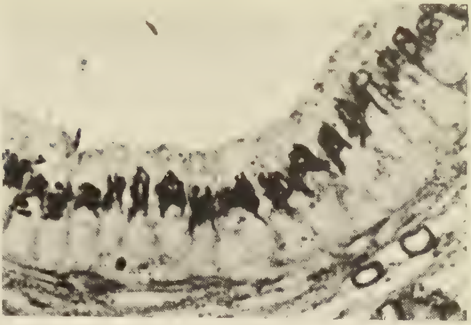


FIG. 2

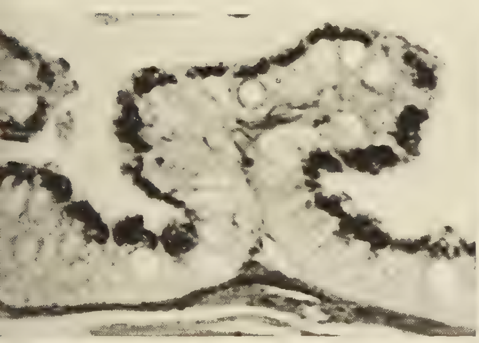


FIG. 3

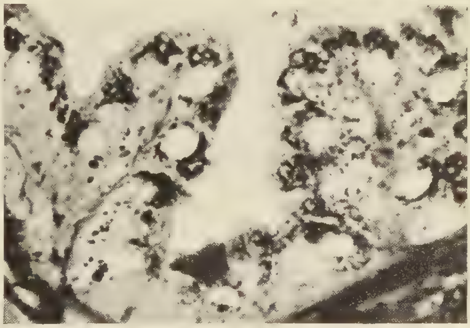


FIG. 4

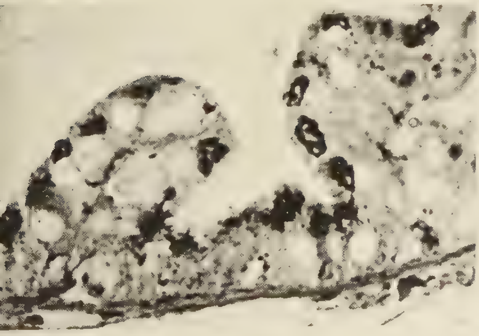


FIG. 5

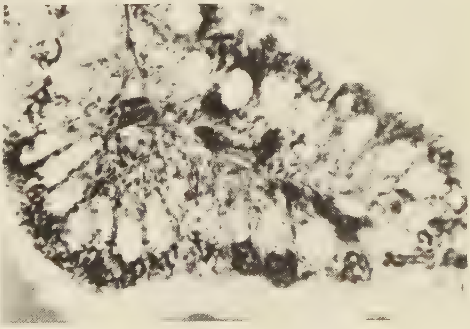


FIG. 6

HORNING—PLATE I

Fig. 3. Dorsal prostatic epithelium from a control mouse. The Golgi substance forms a compact network in the apical cytoplasm.

Fig. 4. Dorsal prostatic epithelium after 8 days' treatment. The Golgi substance has become dispersed.

Fig. 5. Anterior lobe epithelium from a control mouse, showing the Golgi networks which are confined almost entirely to the apical cytoplasm.

Fig. 6. Anterior lobe epithelium after 8 days' treatment. Note the pronounced reaction of the Golgi substance in response to oestrogen. It has become granular and is dispersed throughout the cytoplasm of the epithelial cells which have undergone hypertrophy.

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The Golgi Material of the Neurones of the Central Nervous System of Sheep Infected with Louping-ill

BY

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With sixteen Text-figures

INTRODUCTION

THE histopathology of the nerve-cells of mice and monkeys suffering from louping-ill has been described by Hurst (1931), and an account of the effects of the virus on the neurones of sheep, pigs, and mice has been given by Brownlee and Wilson (1932). The present paper is confined to a description of the changes in the Golgi material of nerve-cells in the brain and spinal cord of sheep infected experimentally with the virus of louping-ill. For comparison the Golgi material of neurones from corresponding regions of the central nervous system of normal animals is described.

Fragmentation of the Golgi material has frequently been observed in degenerating cells and in cells under pathological conditions (Ludford, 1942). The Golgi substance of the epidermal cells of the chick infected with fowl-pox was described by Ludford and Findlay (1926). According to these authors the Golgi material, which in the normal epidermal cell is situated at one pole of the nucleus, becomes hypertrophied during the early stages of infection and in some cases undergoes a reversal of polarity. It breaks up before the virus bodies are fully formed. As the Golgi material is often situated in proximity to the virus bodies, Ludford and Findlay suggest that it may be concerned with the formation or localization of the lipoidal substance deposited around the bodies. Ludford (1928) states that the Golgi substance of the epidermal cells of the cornea of the rabbit infected with vaccinia virus often undergoes hypertrophy, reversal of polarity, and fragmentation. Hypertrophy and fragmentation of the Golgi material is also described by Rio-Hortega (1914) in nerve-cells in a case of paralytic rabies, and by Findlay (1929) in connective tissue cells of rabbits suffering from myxomatosis.

MATERIAL AND METHODS

Small pieces of the cerebrum, cerebellum, medulla oblongata, and the cervical and thoracic regions of the spinal cord of domesticated sheep (*Ovis aries*) suffering from louping-ill were used for the study of the Golgi material. Material from the corresponding regions of the central nervous system of normal sheep was used for comparison. The animals were killed and the tissue dissected out and placed in the fixing fluid as speedily as possible. The material was prepared according to the methods of Aoyama and Da Fano, and sections were cut at 5 μ and 8 μ in thickness. A number of the sections were toned with gold chloride, and of the untoned sections some were stained with Ehrlich's haematoxylin. The cortex and medulla of the Golgi material was more clearly shown in the untoned than in the toned preparations.

The pathological material was obtained from three sheep infected experimentally with the virus of louping-ill. Sheep I and II, both 6 months old, were killed when showing early symptoms of the disease—un-coordinated gait and occasional spasmodic jerks of the head and limbs. Sheep IV, 9 months old, was in a very advanced stage of louping-ill, lying on the floor, and when raised unable to maintain its balance.

The normal material was obtained from Sheep III, over 1 year old, and Sheep V, 2 months old.

We wish to express our thanks to Dr. D. R. Wilson, Animal Diseases Research Association, Moredun Institute, Gilmerton, Edinburgh, who very kindly supplied us with the pathological tissue. Our thanks are also due to Professor James Ritchie for research facilities and for reading the typescript.

OBSERVATIONS

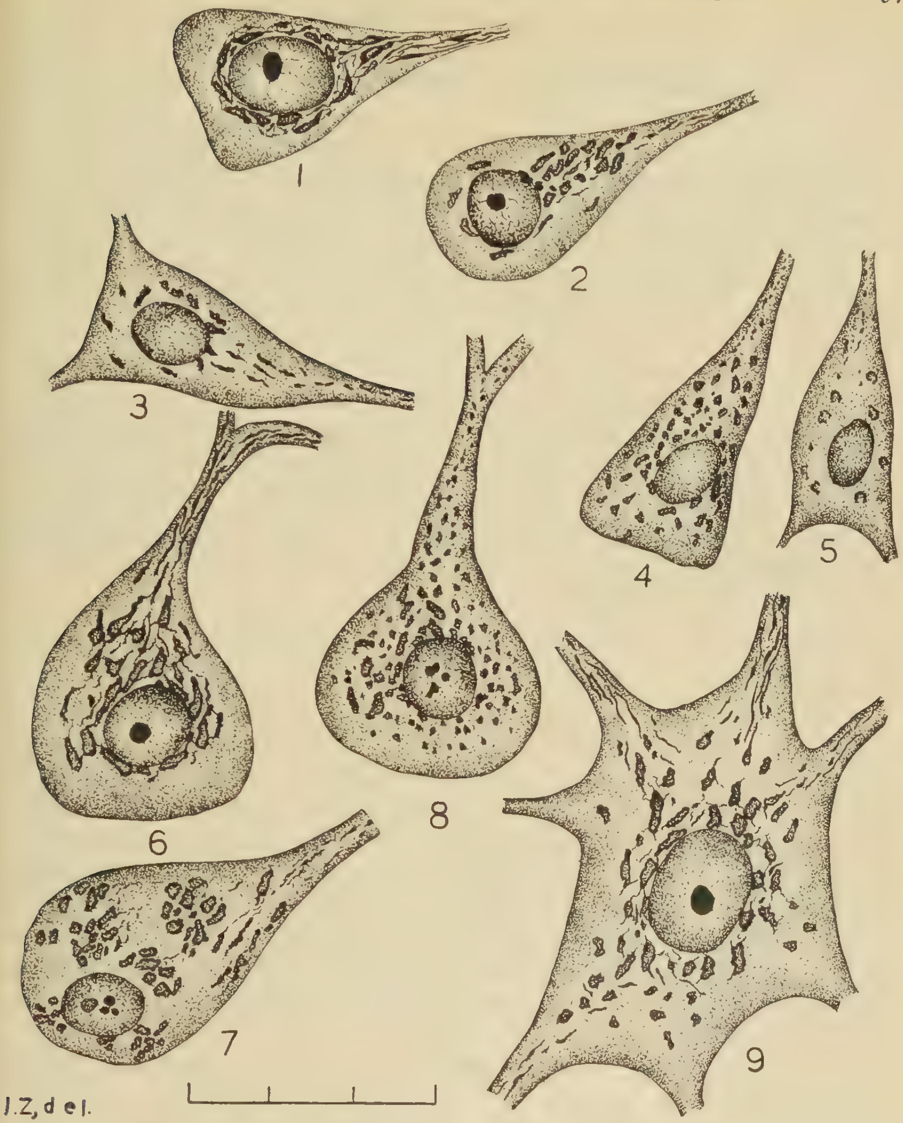
1. *Cerebrum*

Normal sheep

An examination of the pyramidal cells of the cerebral cortex of the sheep showed that the Golgi material is present as filaments, which vary in length, and as irregularly shaped bodies. In some cells the filaments are very numerous, while in others shorter irregular bodies predominate. The Golgi substance surrounds the nucleus and is usually less abundant at the periphery of the body of the cell. It extends for a considerable distance into the cell processes where long filaments are numerous (Text-fig. 1). In many cases the Golgi material seems to be made up of discrete elements which are often in contact with one another. In other cells, particularly in those of the younger animal (Sheep V), it has the appearance of a definite network. The irregularly shaped bodies consist of an argentophil cortex and an argentophobe medulla which is particularly well shown in untoned sections. As the filaments are slender their division into two regions is less clearly visible, but on careful focusing the argentophobe central part can often be made out.

Sheep infected with the Virus of Louping-ill

Sheep I. The distribution of the Golgi material of the pyramidal cells does not differ greatly from that of the normal tissue, except that in some cells it is greater in amount in the region between the nucleus and the apical dendron. Long filaments are, however, less numerous, and in many cells most, or all, of the Golgi substance is in the form of short irregular bodies (Text-fig. 2). Comparison of these neurones with the cortical cells of the normal animals shows that the Golgi material has undergone hypertrophy, that it rarely



All figures from Aoyama preparations.

Fig. 1. Normal sheep. Pyramidal cell of cerebral cortex. Fig. 2. Sheep I. Louping-ill. Pyramidal cell. Fig. 3. Sheep II. Louping-ill. Pyramidal cells. Figs. 4 and 5. Louping-ill. Sheep IV. Pyramidal cells. Fig. 6. Normal sheep. Purkinje cell of cerebellum. Fig. 7. Sheep I. Louping-ill. Purkinje cell. Fig. 8. Sheep IV. Louping-ill. Purkinje cell. Fig. 9. Normal sheep. Multipolar cell of medulla oblongata.

The scale represents a length of 30 μ.

assumes the appearance of a definite network, and that long filaments are reduced in number or are absent (Text-figs. 1 and 2).

Sheep II. The location of the Golgi material is similar to that in Sheep I, except that it is sometimes absent from the cell processes, and is frequently absent or nearly absent from the basal part of the neurone. Filaments, when present, are slender and often appear to be breaking up into smaller bodies (Text-fig. 3).

Sheep IV. According to the form and distribution of the Golgi material the cortical cells are of two types. In one type the Golgi substance is hypertrophied (Text-fig. 4), and in the other it is scanty and in the form of granules and small bodies (Text-fig. 5). In the hypertrophied neurones filaments are few or absent in the cell processes, and when present are always short and granular.

Examination of the pyramidal cells of the infected animals shows that the Golgi material undergoes hypertrophy, and that the filaments become reduced in number. At the same time the Golgi substance is reduced in amount or disappears from the cell processes, and often moves away from the basal part of the cell. Hypertrophy is followed by the fragmentation of the Golgi bodies and by a reduction in the amount of Golgi substance. That all the neurones are not affected equally at the same time is indicated by the presence of hypertrophied cells in the cerebral cortex of Sheep IV. The staining reactions of the cells containing a comparatively small amount of granular Golgi substance indicates that they are necrotic.

2. *Cerebellum*

Normal Sheep

Owing to their large size the Purkinje cells are favourable subjects for investigation. The Golgi material closely surrounds the nucleus and extends laterally towards the periphery and into the basal part of the cell process; it does not extend to the peripheral region in the basal part of the neurone (Text-fig. 6). It consists of long filaments and irregularly shaped bodies, many of which are in contact so as to give the appearance of a network. The filaments are present chiefly in the region anterior to the nucleus, and are all directed towards the cell process into the basal part of which many extend for some distance. The outer deeply argentophil part and the inner argentophobic region are clearly visible in the larger masses of Golgi material.

Sheep infected with the Virus of Louping-ill

Sheep I. Long filaments are less numerous than in normal material, and those present in the cell process often appear to be breaking up to form smaller elements. Irregularly shaped bodies are numerous. In some neurones there is a greater concentration of Golgi substance between the nucleus and the cell process than in those of the normal tissue, and there is often a tendency for several Golgi bodies to lie close together so as to form small groups in the cytoplasm. In many cells the nucleolus is breaking up, and sometimes darkly stained material is present round it (Text-fig. 7).

Sheep II. The Golgi material is similar to that of the Purkinje cells of Sheep I, except that the grouping of several Golgi elements together is less pronounced.

Sheep IV. Golgi material is present as irregular masses. Long filaments are absent and comparatively little Golgi substance is present in the process (Text-fig. 8). In some cells the Golgi bodies are small and are probably formed by fragmentation of larger masses.

It is concluded that the first visible results of infection of the Purkinje cells are hypertrophy of the Golgi material, the disappearance of long filaments, a reduction in the amount of Golgi substance in the cell process, a tendency to a concentration of Golgi material anterior to the nucleus, and for a grouping of Golgi bodies in the cytoplasm. This is followed by fragmentation.

3. *Medulla Oblongata*

Normal Sheep

The following account is based upon the examination of the large multipolar nerve-cells of the medulla oblongata, which contain a large number of filamentous and irregularly shaped Golgi bodies. As in the normal cells of the cerebrum and the cerebellum, many of the bodies seem to be joined to form a network, and filaments extend into the cell processes (Text-fig. 9). In the cells of the medulla of the lamb (Sheep V) the Golgi material is greater in amount than in the older animal; it often more closely surrounds the nucleus and is concentrated in the neighbourhood of one or more of the cell processes (Text-fig. 10). The Golgi elements are similar in appearance to those of the cerebrum and cerebellum.

Sheep infected with the Virus of Louping-ill

Sheep I. In some of the multipolar cells numerous long filaments are present, while in others most of the Golgi substance is in the form of irregular bodies. Filaments are more numerous in the basal part of the cell processes than elsewhere, but in many cases the Golgi material in these regions is considerably less than in the corresponding parts of the normal cells (Text-fig. 11).

Sheep II. Most of the neurones are similar to the cells of Sheep I, but in some cases the large masses of Golgi material appear to be breaking up.

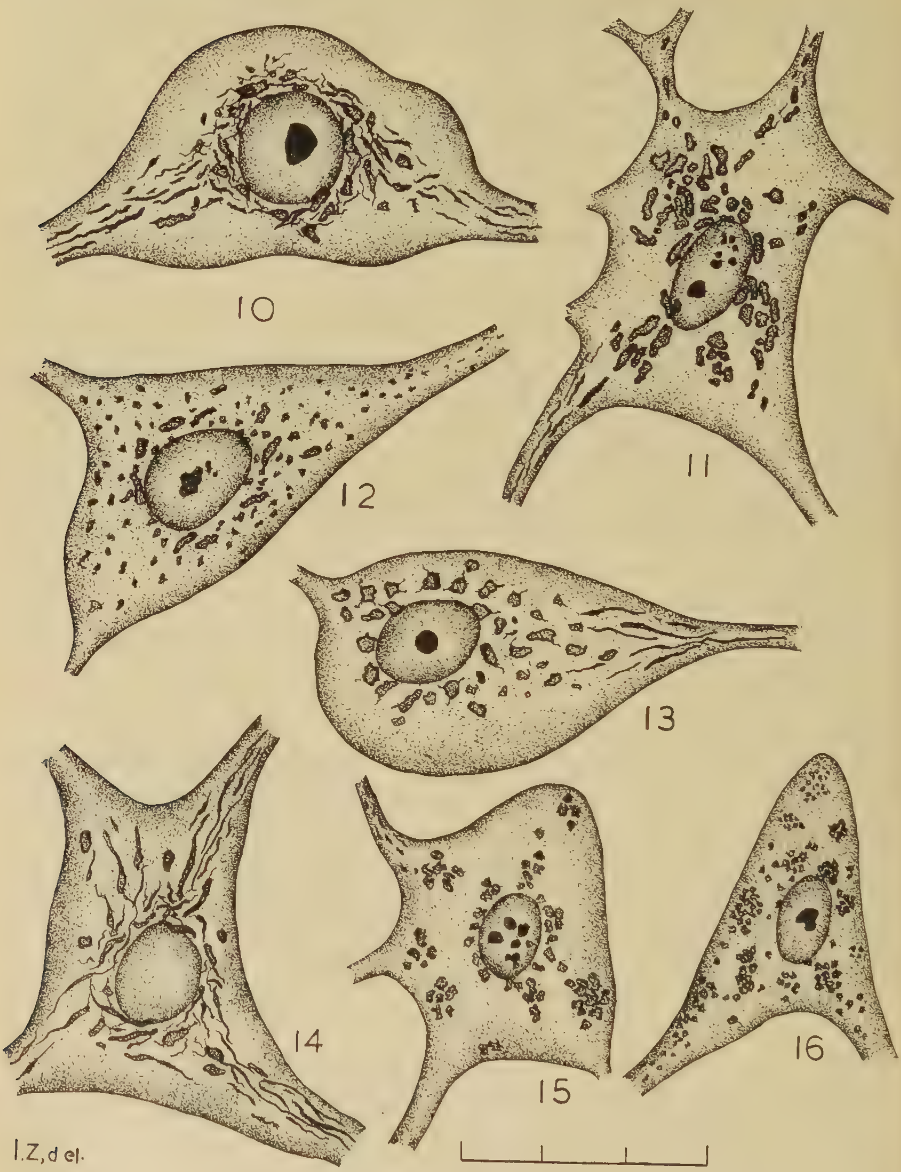
Sheep IV. The Golgi bodies are smaller than in normal cells. Short filaments may be present in the processes but are absent from other parts of the neurones (Text-fig. 12).

The Golgi material of the infected multipolar nerve-cells of the medulla undergoes hypertrophy which is not so marked as in the pyramidal cells of the cerebral cortex and the Purkinje cells of the cerebellum. The chief effects of the virus seem to be the disappearance of long filaments, a reduction in the amount of Golgi substance in the processes, and finally fragmentation of the Golgi bodies.

4. *Spinal Cord*

Normal Sheep

The multipolar motor nerve-cells of the ventral horns were chiefly used for this part of the study. Cells in other parts of the cord were also examined,



All figures from Aoyama preparations.

Fig. 10. Lamb (Sheep V). Multipolar cell of medulla. Fig. 11. Sheep I. Louping-ill. Multipolar cell of medulla. Fig. 12. Sheep IV. Louping-ill. Multipolar cell of medulla. Figs. 13 and 14. Normal sheep. Motor nerve-cells of ventral horn of spinal cord. Fig. 15. Sheep I. Louping-ill. Motor nerve-cell of ventral horn of spinal cord. Fig. 16. Sheep IV. Louping-ill. Motor nerve-cell of ventral horn of spinal cord.

The scale represents a length of 30 μ.

and the distribution and structure of the Golgi material was found to be similar to that in the cells of the ventral horns.

The form and arrangement of the Golgi material varies somewhat in different cells. In some of the neurones irregularly shaped bodies predominate and in others filaments are very numerous. In a few cases the Golgi substance surrounds the nucleus and appears to form a network. Filaments are present in the basal region of the axon and the dendrons (Text-figs. 13 and 14).

Sheep infected with the Virus of Louping-ill

Sheep I. The Golgi material of some of the motor cells of the ventral horn is hypertrophied and is fairly evenly distributed through the cytoplasm; filaments are not numerous. In a large number of neurones it closely surrounds the nucleus, or forms several groups scattered through the cell. Filaments are short or absent, and the irregularly shaped Golgi bodies are smaller than in normal tissue. The Golgi material is reduced in amount or is absent in the majority of the cell processes. Several small nucleoli are frequently present (Text-fig. 15).

Sheep II. The distribution and structure of the Golgi material is similar to that of Sheep I.

Sheep IV. The Golgi substance of some of the motor nerve-cells is hypertrophied, but in most cases it consists of comparatively small bodies either concentrated about the nucleus or else situated in small groups distributed through the cytoplasm. It is often absent from the axon and the dendrons (Text-fig. 16).

The changes in the morphology and distribution of the Golgi substance within infected motor nerve-cells of the ventral horns is very marked. The Golgi material varies in distribution, but in all the cells examined it consists of bodies which are smaller than those of the normal tissue, and a net-like structure is never present. The first visible change in the Golgi material is hypertrophy, and this is followed by a grouping of the Golgi elements and their fragmentation to form smaller bodies. Infection is also followed by a breaking up of the large filaments of the normal cell, and the reduction and disappearance of the Golgi substance in the axon and in the dendrons.

DISCUSSION

Observations on the neurones of the central nervous system of normal and infected sheep show that the Golgi material undergoes changes consequent upon the invasion of the cells by the virus of louping-ill. In all the types of infected nerve-cells examined by the writers the Golgi substance hypertrophies and finally breaks up. The main visible changes are, therefore, similar to those described by Rio-Hortega (1914), Findlay (1929), Ludford and Findlay (1926), and by Ludford (1928) as due to the invasion of cells by other viruses. In addition certain other features of the behaviour of the Golgi material are noted.

The cells of the spinal cord and the pyramidal cells of the cerebral cortex show the greatest changes. The Golgi material of the Purkinje cells of the cerebellum differs considerably from the normal, but not to the same degree as in the neurones of the cerebrum and spinal cord. The Golgi material of the multipolar cells of the medulla does not undergo marked hypertrophy, but, like that of the other cells investigated, finally breaks up. In the neurones from the different regions the Golgi substance in the cell-processes becomes reduced in amount and in some cases disappears completely. In the cerebellum and spinal cord the Golgi elements often form groups scattered through the cytoplasm. The Golgi material tends to move away from the basal part of infected cells of the cerebrum and cerebellum. Certain cells in the cerebellum, medulla, and spinal cord of the infected animals, particularly in Sheep IV, contain numerous argentophil granules. As some of these cells stain deeply with haematoxylin and others stain very faintly, it was not possible to determine with certainty if they are necrotic or imperfectly preserved by the silver technique. If the cells are necrotic the argentophil granules may represent a later stage of fragmentation of the Golgi material than is described in the preceding sections of this paper.

Hurst (1931) found that in experimental louping-ill of the monkey the Purkinje cells of the cerebellum were severely affected, but that in the mouse necrosis may be slight. Brownlee and Wilson (1932), describing the histopathology of the nervous system, observe that necrotic neurones were not identified in the cerebral cortex of sheep infected experimentally with louping-ill, but were present in two natural cases. In both experimental and natural cases a variable number of Purkinje cells were always destroyed. Necrotic nerve-cells were also observed in the medulla and spinal cord. In the pig there was relatively little destruction of the nerve-cells, while in the mouse the principal lesion was necrosis of most of the large neurones of the medulla and spinal cord. The observation of Brownlee and Wilson that necrotic cells are not present in the cerebral cortex of experimental sheep, but are present in the cerebellum, medulla, and spinal cord, suggests that the cells containing argentophil granules present in the animals described in the present paper are necrotic and in a late stage of the fragmentation of the Golgi material. The writers found that the Golgi substance was not always at the same stage of degeneration in all the cells of a particular region of the brain or spinal cord. This agrees with previous work on the histopathology of louping-ill.

On comparing the Golgi material of the neurones of the brain and spinal cord of the lamb (Sheep V) with similar cells of the normal adult sheep it was found that in the lamb a greater number of cells possessed Golgi substance which appeared to form a network around the nucleus, and which was often composed of very thick strands. Andrew (1939) claims that in the nerve-cells of young mice the Golgi material consists of large oval bodies connected by short threads to form a reticulum. In older animals the bodies connected by threads are smaller, and in some cases threads are absent. In old mice the Golgi material is present as granules scattered through the cytoplasm.

Many cytologists believe that the Golgi material is always present as separate bodies and that the appearance of a network is an artifact (Bourne, 1942). In the nerve-cells of the sheep it is impossible to determine with certainty if a network sometimes exists. The occurrence of separate bodies in a large number of the cells of the normal sheep examined suggests that the Golgi substance is always present as discrete elements which under certain conditions may be situated close together in the cytoplasm.

SUMMARY

1. The Golgi material of the pyramidal cells of the cerebral cortex, the Purkinje cells of the cerebellum, and the multipolar cells of the medulla oblongata and ventral horns of the spinal cord of the sheep is present as filaments and as irregularly shaped bodies. In some of the cells, particularly in the lamb (Sheep V), the Golgi material has the appearance of a network. As it is frequently present as separate bodies it is suggested that it may always consist of discrete Golgi elements which are sometimes situated in close proximity or in contact with one another. Filamentous Golgi elements are present in the basal part of the cell processes.

2. An examination of neurones from the corresponding regions of the central nervous system of sheep infected experimentally with louping-ill showed that the Golgi material undergoes changes consequent upon the invasion of the cells by the virus. The Golgi material undergoes hypertrophy, and at the same time there is a reduction in the number of filamentous Golgi elements and a reduction in the amount of Golgi substance present in the cell processes. These changes are followed by fragmentation. All the neurones of a particular region are not affected equally at the same time. The Golgi material of the Purkinje cells tends to form groups in the cytoplasm prior to fragmentation. In the multipolar cells of the medulla oblongata the hypertrophy of the Golgi material is not as great as in the other regions of the central nervous system. The Golgi material of the motor nerve-cells of the ventral horns of the spinal cord undergoes considerable hypertrophy which is followed by a grouping of the Golgi elements and fragmentation.

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A Quantitative Study of the Osmium Impregnation of the Contractile Vacuole of *Chilomonas paramecium* (Cryptomonadina)

BY

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With two Text-figures

INTRODUCTION

THE application of the metazoan Golgi techniques of Weigl (Mann-Kopsch) and Kolatchev to Protozoa has shown that the region immediately surrounding the contractile vacuole in many organisms is very osmiophilic. This was first shown by Nassonov (1924, 1925), who demonstrated the presence of osmiophil cortices around or associated with the contractile vacuoles of *Paramecium caudatum*, *Lionotus folium*, *Nassula laterita*, *Dogielella* sp., *Childon* sp., *Campanella umbellaria*, *Epistylis gallea*, *Zoothamnium arbuscula*, *Vorticella* sp., and *Chilomonas paramecium*. Nassonov attempted to homologize the osmiophil material and the contractile vacuole in these forms with the Golgi apparatus in metazoan cells—a hypothesis which was only accepted in part by later workers.

The osmium techniques have since been applied to a great many different Protozoa, and a vast amount of literature has been published on the whole question of the homology of the Golgi apparatus in Protozoa; this problem has recently been reviewed in detail (Smyth, 1944) and will not be discussed further here.

Apart from the question of homology of the Golgi apparatus, the fact that there is in many Protozoa a ring of osmiophil material surrounding the contractile vacuole is in itself of interest. There is much evidence from the literature of the problem to show that in the hands of different workers the osmium techniques have produced very different results—even in work on the same organism. Any cytologist who has worked constantly with the osmium technique is aware of the fact that it is very inconsistent in its results, sometimes giving beautiful preparations, at others failing to impregnate completely. Gatenby (1941) writes: 'As regards the technique, it must be at once admitted that it can be capricious. The reason or reasons for this are unknown to cytological technicians.'

It is not surprising then to find, that with work based on such a fallible echnique, discrepancies in the results of different workers are common. For example, in the case of the Cryptomonad, *Chilomonas paramecium*, Nassonov (1924) impregnated the contractile vacuole with osmium, but did

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not mention whether his preparations were always successful; in the same organism Hall (1930) described the contractile vacuole wall blackened in only 54 per cent. of the specimens; Gatenby and Smyth (1940) stated that in normal undividing cultures of *Chilomonas* the contractile vacuole was impregnated in 99 per cent. of the organisms examined; Patten and Beams, however, were unable to impregnate the contractile vacuole in this organism, though specimens of *Euglena* in the same culture solution were successfully blackened. Similarly, in *Colpidium colpoda* the present writer (1941) showed that the contractile vacuole in this form has a very well-marked osmiophilic cortex, the presence of which had been previously denied by Hall and Alvey (1933) using essentially the same technique.

Numerous other examples could be quoted from the literature of protozoan cytology, but those given above suffice to show that there is a need for an investigation into the factors governing osmium impregnation, and that until these factors are fully understood it seems likely that further confusion will only arise in future work on this problem.

In the present paper a preliminary investigation into some of the possible factors governing impregnation of the contractile vacuole in *Chilomonas paramecium* is described. This organism has the advantage that it has been investigated cytologically by several workers and its general morphology is consequently well known; it is easy to obtain in almost pure cultures which can be maintained for some considerable time without difficulty, and it is sufficiently large to enable its contractile vacuole and related structures to be observed without difficulty.

PREVIOUS WORK

Only one attempt has been made to throw light on the osmic impregnation of Protozoa by a quantitative investigation. MacLennan (1940) investigated the impregnation of the contractile vacuoles of *Actinosphaerium eichhorni*, *Epidinium caudatum*, *Eudiplodinium maggii*, *Haptophrya michiganensis*, *Ichthyophthirius multifiliis*, *Metadinium medium*, and *Ostracodinium monolobum*. Only in *Haptophrya* and *Metadinium* was the contractile vacuole impregnated in 100 per cent. of the organisms examined. In the remaining ciliates the percentage impregnation was considerably lower, varying between 33 and 64 per cent. MacLennan attempted to explain the inconsistency of impregnation of the contractile vacuole in these forms as being due to the fact that the osmiophily varies with the phase of the vacuole. He states that 'the impregnation of the contractile vacuoles is consistent *when like functional stages are compared* and the apparent inconsistency in impregnation shown in Table 1 (MacLennan's paper) is due to lack of analysis of the data'. He claims that the quantitative variation as shown by his results is correlated with the 'cyclic granular aggregations demonstrated in living specimens'. The 'granular aggregations' referred to by MacLennan when concentrated around the contractile vacuole are considered to represent the so-called 'osmiophil cortex' of Protozoa; he considers there is no true osmiophil vacuolar membrane in any of the forms studied except *Haptophrya.* Although MacLennan states, 'The slides were searched systematically with the aid of a mechanical stage and all the individuals of the species in question were studied and the impregnation recorded', no details of the numbers of organisms studied were given, nor does he give any statistical evidence as to the accuracy of his results.

MATERIAL AND METHODS

An account of the Weigl method as used for Protozoa has already been given in a previous paper (Gatenby and Smyth, 1940), but a slight variation of the usual technique is used in the present work and will be described here in some detail.

The organisms were obtained from an infusion of soil and leaves to which some boiled hay solution was added. Unlike most flagellates common in hay infusions, *Chilomonas paramecium* will remain in large numbers in such a culture—apparently being able to withstand quite large changes in pH. A good culture will remain in a flourishing condition for several months, provided a little fresh tap-water is added to keep up the water-level, and the culture is covered to keep bacterial pollution at a low level. The organisms were concentrated by gentle centrifuging in a hand centrifuge. After concentration the flagellates were shaken up to break up any clumps and were allowed to remain in about $\frac{1}{4}$ in. of solution in the centrifuge tube for half an hour to allow any cytological disturbances caused by centrifuging to subside. Fixation with Mann's fluid followed. In order to keep uniformity throughout the series of experiments, the amount of fixative was kept constant—3 c.c. were used in every case. This was introduced into the centrifuge tube by means of a fine pipette—the fixative being squirted in very suddenly to produce as instantaneous and uniform a fixation as possible. The tube was then shaken further for a few minutes to complete the mixing. After fixation the organisms were washed in two changes of distilled water—15 minutes each—brought into 3 c.c. of pure osmium tetroxide solution, and transferred to an osmication tube and placed in an oven at 32° C. Details of the tubes and the fixation varied in different experiments and will be described under the various sections. After osmication was completed, or, in some experiments, during the process of osmication, the organisms were removed from the osmium tetroxide with a fine pipette and mounted in a drop of Farrants's medium. This latter mounting medium allows of surprisingly clear cytological observation of the osmicated Protozoa, and has the advantage that it enables a very small number of the flagellates to be removed and examined—a procedure which is difficult if they have to be dehydrated, cleared, and mounted in balsam. The percentage of impregnated organisms was counted carefully by observation under oil immersion, the field being moved uniformly by means of a mechanical stage. In each preparation the number of impregnated organisms in the first 500 observed were taken.

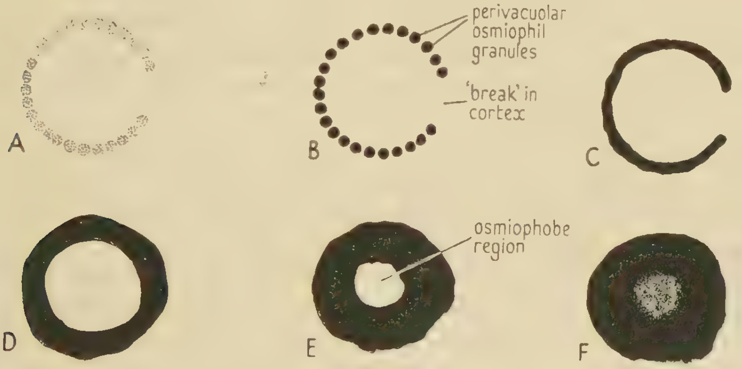
MORPHOLOGY OF CHILOMONAS PARAMECIUM

A detailed account of the morphology of this Cryptomonad has already been given in a previous paper (Gatenby and Smyth, 1940) and only a brief description will be included here. The organism is about 20 μ in length, but the size varies considerably with the state of nutrition. The nucleus is spherical and median with a large nucleolus. There are two anterior flagella. The gullet is deep and contains peripheral trichocysts. The single contractile vacuole is anteriorly placed, and lying between it and the nucleus are two (in old cultures) or one (in rapidly dividing cultures) large ovate endoplasmic bodies, which have been identified as pyrenoids. In addition to the osmiophil material associated with the contractile vacuole, a number of small scattered osmiophil granules are invariably found in the region between the vacuole and the pyrenoids.

THE STAGES OF IMPREGNATION

Examination of preparations made at intervals during the osmicating process showed that the impregnation of the contractile vacuole of *Chilomonas* follows a very definite course, which, for the sake of description, can be divided into a number of more or less well-defined stages. The earliest sign of impregnation is the appearance around the contractile vacuole of a ring of light greyish-coloured granules of almost uniform size (A, Text-fig. 1). These granules follow the perimeter of the contractile vacuole very closely, but as far as could be observed do not lie on or in any distinct vacuolar membrane. This ring of granules is complete except for a small break in its periphery, equal in length to about one-sixth of its circumference. The position of this gap is always the same, i.e. it lies on the side of the contractile vacuole nearest to the gullet. The area between the contractile vacuole and the pyrenoids is occupied by a few scattered greyish granules of approximately the same size as those surrounding the cortex.

At a slightly later stage (B, Text-fig. 1) the granules around the vacuole become more heavily impregnated and losing their greyish colour become now a very dense black. At the next stage (C, Text-fig. 1), the material around the vacuole loses its granular nature and appears as a definite osmiophilic ring, which, however, still shows the peripheral break in most cases. Its granular origin is evident from the wavy irregularity of its outline, and the fact that stages intermediate between B and C are easily found in all preparations. As osmication proceeds, the impregnation of the vacuole becomes increasingly heavier and the thin wall found in stage B becomes thickened to form a dense osmiophilic cortex with a small osmiophobic area in its centre (D and E, Text-fig. 1). In preparations osmicated for sufficient time to show both these stages, the peripheral break is seldom visible; there is little doubt from the appearance of the earlier stages that this gap in the cortex becomes obliterated by the over-impregnation of the osmium. Prolonged osmication results in the complete impregnation of the whole contractile vacuole region, and an apparently solid mass of osmiophil material showing an irregular outline is obtained (F, Text-fig. 1). The impregnation of the central (osmiophobe in earlier stages) area of the vacuole varies quite considerably in this final stage, and is seldom actually as heavy as the cortical region, though this appears to be the case at first sight. By using a very strong and concentrated source of illumination, this central region in some cases can be seen to be made up of a thin osmiophil layer which is in the nature of a membrane lying *within* the more heavily osmiophilic outer layer. In other cases, however, the central region appears to be as heavily impregnated as the outer cortex, and is equally opaque even with very intense light.



TEXT-FIG. 1. Stages in the impregnation of the osmiophil cortex of *Chilomonas paramecium*. Somewhat diagrammatic.

Although the course of impregnation is relatively easy to follow and interpret, it must be emphasized that the various stages outlined above by no means complete the picture, for since the process of impregnation is a continuous one, as is to be expected, a number of stages intermediate between those described above are found in all preparations. Moreover, the degree of impregnation in any culture after a given time of incubation is not by any means uniform, and a number of different stages can be seen in any one preparation.

An analysis of the percentage of different stages found in a typical culture (using sample A OsO_4) showing 70 per cent. maximum impregnation provides some interesting results. After 16 hours' incubation the first five stages A to E are all visible; about 64 per cent. show stages B or C and only some 17 per cent. are in the earliest impregnation phase. Approximately 16 per cent. have progressed to stage D, and a very small number (3 per cent.) show stage E. The complete impregnation of the vacuole (stage F) is never shown in organisms osmicated for less than 30 hours. After 27 hours' incubation the distribution of the stages follows the course expected, namely, there is a decrease in A, B, and C, which is compensated by a marked increase in D and E. After 66 hours stage E reaches its peak and large numbers have reached the total impregnation stage F, while the earlier stages A to D are much less frequent. Further osmication shows that numbers of organisms previously at stage E have been converted to F, and in the final observation (162 hours) two-thirds of the impregnated organisms are at the F stage, 21 per cent. are at stage E, and only a very small proportion of the earlier stages (total 12 per cent.) can still be found.

At all stages of impregnation the scattered osmiophil granules—the first appearance of which was noted in the earliest stage—were present. Apart from becoming more densely blackened, their appearance changed little during the later period of impregnation.

IMPREGNATION UNDER NORMAL CONDITIONS

Normal conditions are those described under 'Methods' in the earlier sections. The first set of experiments were designed to determine whether under uniform conditions of fixation and osmication the percentage of organisms that are impregnated is constant.

The tubes used to carry out the osmication in this series of experiments were of ordinary drawn glass with measurements of 5 × 1 cm. Before use they were carefully cleaned first with soap and water, secondly with chromic acid, and finally rinsed in tap-water followed by distilled water. Fixation time was kept constant at 60 minutes. In one series of experiments a number of organisms were fixed simultaneously in the same centrifuge tube, and, after washing, were divided into three parts and transferred to three separate tubes for osmication. A second series of organisms was treated in *separate* centrifuge tubes, and after washing transferred to three separate tubes. Two samples of osmium tetroxide, A and C, obtained from different manufacturers, were used. In previous cytological studies on Protozoa, A had been found to give good impregnation results and C poor results, but the samples had not been tested quantitatively.

The results of a series of experiments of this nature are given in Table 1. The differences between the figures of the percentage osmication with the two samples of osmium tetroxide were in all cases highly significant, the impregnation with A being nearly twice that obtained with C. Organisms with common fixation and separate osmication showed a high degree of uniformity of impregnation, with standard deviations of 0·85 and 1·22 for A and 2·35 and 0·47 for C. It must be noted, however, that although the impregnation of any group of three tubes with a common fixation was fairly uniform, the difference in the figures for any two experiments of the same type with the same sample of OsO_4 was significant, i.e. the results of any one experiment were not reproducible. Cultures, both fixed and osmicated separately, showed standard deviations between the three tubes—in three out of the four experiments—that were significantly higher than cultures with a common fixation.

In order to investigate more fully the process of impregnation under normal conditions the rate of impregnation of vacuole of two cultures using the two samples of osmium tetroxide was determined. The procedure adopted was identical with that described previously for investigating the stages of impregnation—namely, samples were taken at intervals during the osmication process and the percentage of impregnated organisms—in this case independently of their stages—were counted.

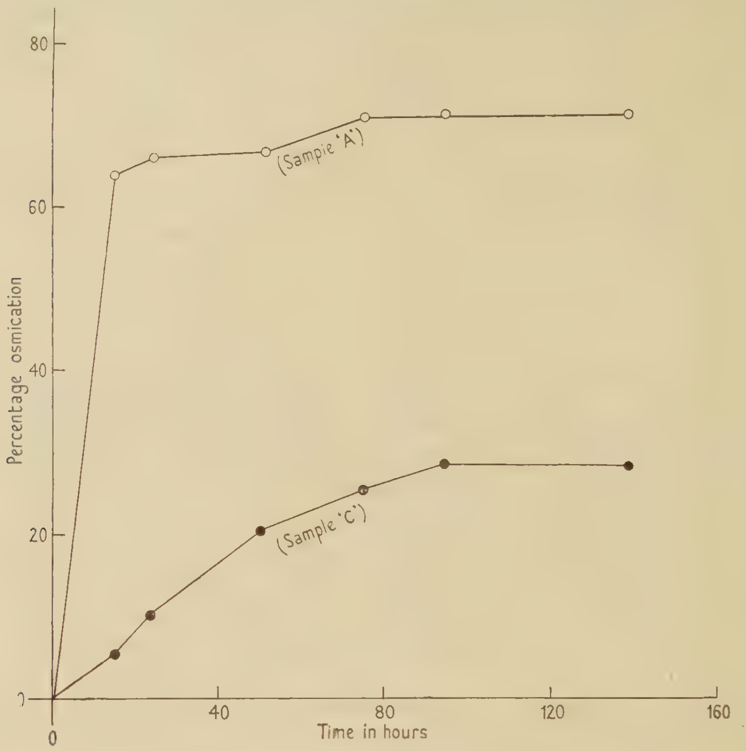
TABLE 1. *Percentage impregnation after common and separate fixation. Two different samples of osmium tetroxide used for osmication*

OsO_4 *sample*	*Fixation*	*Per cent. impregnation*	*Mean*	*Standard deviation*	*Standard error*
A	Common	84·0 82·5 84·0	83·67	0·85	0·49
A	Common	71·0 70·5 73·0	71·5	1·22	0·71
C	Common	30·0 25·0 25·0	26·66	2·35	1·36
C	Common	31·0 31·0 30·0	30·66	0·47	0·27
A	Separate	70·0 75·0 85·0	76·66	6·24	3·60
A	Separate	74·0 70·0 62·0	68·66	5·46	3·15
C	Separate	35·0 30·0 30·0	31·66	2·36	1·36
C	Separate	30·0 33·0 36·0	33·0	2·45	1·42

The results are shown graphically in Text-fig. 2. With sample A the maximum impregnation is almost reached within 16 hours, i.e. the majority of vacuoles which will be finally impregnated become impregnated within this time. With the poorer sample of osmium tetroxide—sample C—the initial impregnation rate is slow, but the percentage impregnation rises slowly with time, to settle to a steady figure in about the same time (approx. 100 hours) as sample A. From these curves it is at once evident that prolonged osmication cannot increase the percentage of impregnated organisms in any single culture.

EFFECT OF MIXING ON IMPREGNATION

During the course of examination and counting large numbers of preparations of osmicated *Chilomonas*, it was noted that in the case of very poorly impregnated cultures, i.e. cultures showing less than 30 per cent. impregnation, the impregnated organisms appeared sometimes in clumps in the field of the microscope. This suggested the possibility that fixation or osmication, or both, was not uniform throughout the culture solution, and that some parts had been better fixed than others or were in closer contact with the osmium tetroxide solution during the incubation.



TEXT-FIG. 2. Rate of impregnation of the osmiophil cortex of *Chilomonas paramecium* with two samples of osmium tetroxide.

In order to eliminate any possible error due to this cause, experiments were carried out in which the solutions were kept in constant motion during the entire processes of fixation and osmication. The tubes containing the organisms were placed in a small box fitted to the axle of an electric motor, which rotated five times a second—a speed sufficient to mix the contents thoroughly and yet not centrifuge them to one end of the tube. The organisms were rotated during fixation, washing, and osmication—in the latter case the whole arrangement being fitted into the incubation oven.

The results obtained are shown in Table 2. The percentage impregnation showed a much greater variation with both specimens of osmium tetroxide than when osmicated under normal conditions. This was especially noticeable with sample A, when the impregnation figures in three experiments fell well below 30 per cent.—a low level never obtained in normal experiments with this sample. In the first set of experiments of this nature ordinary corked tubes were used as before, and it was noted that since the corks came into contact with the osmium solution more frequently as a result of rotation, the former became more heavily blackened than was usual, and it was thought that this might possibly have some effect on the impregnation. In order to eliminate any factor introduced by this the experiments were repeated with tubes that had been sealed completely by means of a fine hot gas flame. Although some of the individual results were higher than those obtained with corked tubes, the variation between the percentage impregnation for a given specimen of osmium tetroxide was still considerable and in no way compared with the more uniform results obtained by using stationary tubes. It is interesting to note, however, that in one case of osmication with C, the effect of rotation gave an impregnation figure of 70 per cent.—a result considerably higher than any previously obtained with this sample in stationary tubes, and comparable to that obtained with the better sample of the osmium tetroxide.

TABLE 2. *Percentage impregnation after continual mixing during fixation and osmication, using corked and sealed tubes, with two specimens of osmium tetroxide*

	OsO_4 'A'		OsO_4 'C'	
	Corked tubes	*Sealed tubes*	*Corked tubes*	*Sealed tubes*
	78·0	41·0	40·0	4·0
	61·0	38·0	29·0	30·5
	25·0	85·0	30·0	26·5
	93·0	90·0	17·5	34·0
	20·0	17·0	15·5	16·0
	17·5	21·0	23·0	70·0
Standard deviation	30·0	25·4	8·3	20·4

EFFECT OF FIXATION TIME ON IMPREGNATION

Organisms were treated as for normal osmication, but fixed for different periods of 5, 30, 60, and 90 minutes, both samples of osmium tetroxide being used.

Results of the four experiments carried out are shown in Table 3. It is at once evident that with either sample of the osmium tetroxide the impregnation shows no correlation with the fixation time, and that the variation between the impregnations obtained with either A or C for different fixation times is only that approximately to be expected under normal conditions as shown in Table 1, and as far as can be determined the period of fixation—within the limits of the above experiments—has no effect on the final impregnation.

TABLE 3. *Percentage impregnation for different fixation times, using two specimens of osmium tetroxide for osmication*

Fixation time in minutes	OsO_4 'A'		OsO_4 'C'	
	Exp. 1.1	*Exp. 1.2*	*Exp. 1.3*	*Exp. 1.4*
5	70·0	76·5	33·0	30·5
15	71·0	73·0	40·0	30·0
30	80·0	66·0	31·0	30·0
60	85·0	70·5	31·5	36·0
90	83·0	63·0	36·0	34·0

DISCUSSION

The cytological pictures presented by the contractile vacuole of *Chilomonas* during the various stages of its impregnation are difficult to interpret. The appearance of the osmium precipitate in the form of granules suggests that the so-called osmiophil cortex may be granular in origin. The occurrence of perivacuolar osmiophil 'granules' has been noted previously in a few other Protozoa. In the *Ophryoscolecidiae* MacLennan (1933) has claimed that the warm method of impregnation produced a thick osmiophil membrane around the contractile vacuole, whereas the longer method at room temperature demonstrated the same region to be granular. He also suggested that since the solid cortices around the contractile vacuoles of forms such as *Chilodon* and *Dogielella*, as figured by Nassonov (1924, 1925), showed a distinct granular roughening in the outer region, the solid structures described by him are really artifacts produced by over-osmication.

In *Plagiopyla* (Smyth, 1941) the impregnation of the contractile vacuole goes through a series of stages almost identical with those described in *Chilomonas*: 'The osmiophil cortex first makes its appearance as a thin ring of black beads. On further impregnation the beads coalesce, and the typical osmiophil cortex is produced. Further impregnation makes the ring so thick that the whole structure appears solid.' The phases in this form were not investigated in any great detail, and no figures are available. In *Lagenophrys* (quoted by Gatenby, 1941) Willis has described 'blackening of the accumulated granules which give the appearance of a distinctive Golgi cortex to the contractile vacuole'. Gatenby (1941) has shown that in *Vorticella* during binary fission the osmiophil cortex of the parent cell passes over completely to one daughter organism, whereas a new one is formed in the other by the accumulation of scattered osmiophil granules around the contractile vacuole.

On the other hand, it must be emphasized that there is some evidence from metazoan cytology that granular precipitates of heavy metals—notably silver —may not necessarily indicate the presence of pre-formed granules. Barnett and Fisher (1943), using the acid silver nitrate method to demonstrate the presence of vitamin C in artificial mixtures of olive oil, ground glass, or kieselguhr in gelatine solutions, found that the form of the silver precipitate had 'no bearing on the prior localisation of ascorbic acid' and concluded that 'it is unjustifiable to infer the whereabouts of ascorbic acid within the cell from the site of the silver precipitates by the silver nitrate method'. Bourne (1944), however, has criticized their results and has cited cases where exact correspondence was obtained between granular mitochondria stained in Janus green B and vitamin C granules in adjacent frozen sections of various tissues (Bourne, 1935; Leblond, 1934; Giroud, 1938). Thus, in some cases at least, metallic precipitates in the form of granules would seem to indicate the presence of pre-formed granules. Nevertheless, the perivacuolar 'granules' in *Chilomonas* have never been demonstrated by methods other than those based on the reduction of osmium tetroxide, and the possibility that these osmiophil granules merely represent the first stage in the reduction of the osmium tetroxide in a specialized area, and not necessarily pre-formed granules, must therefore not be overlooked.

Nassonov (1924) figures the 'broken-ring' type of osmiophil cortex in *Chilomonas* as representing that of a contractile vacuole in diastole, and considered the completely impregnated vacuole to represent the condition found at *systole* by the collapsing of the osmiophil cortex. From the fact that in the present experiments the very early impregnation stages (about 16 hours) showed only open rings, whereas the very prolonged stages showed mainly completely impregnated vacuolar areas, it must be concluded that the so-called 'systole' condition of Nassonov is simply due to over-impregnation, and that the osmiophil material in *Chilomonas* does not change during the vacuolar cycle.

Since the contractile vacuole itself undergoes collapse, whereas the osmiophil cortex does not, it is evident that the latter lies *outside* the region of the contractile vacuole proper. Some workers have taken the impregnation of the vacuolar region as being indicative of the presence of a vacuolar membrane, but if the osmiophilic area lies *outside* the vacuole, this provides no evidence for the existence of such a membrane.

The presence of a 'break' in the osmiophil ring in the early stages of impregnation suggests the presence of a permanent pore by means of which the contractile vacuole is enabled to discharge its contents to the exterior. According to MacLennan (1944), a permanent pore exists in the contractile vacuole region of the *Ophryoscolecidae* where a similar 'break' is found, whereas in *Amoeba proteus*—where there is no perivacuolar osmiophil material (Singh, 1938)—the vacuolar pore is lacking.

The results of the quantitative experiments on the degree of impregnation show that the percentage impregnation with the same specimen of osmium tetroxide and the same fixing fluid can vary quite considerably even under the most carefully controlled conditions. The fact that organisms fixed in the same tube and osmicated separately give much more uniform results than organisms fixed separately, indicates that there is some controlling factor introduced *at the time of fixation.* The only materials concerned in the fixation process are (*a*) the culture solution containing the organisms; (*b*) the fixing fluid; (*c*) the centrifuge tube. Now since both (*a*) and (*c*) are identical in any series of experiments, the factor must be introduced by either the centrifuge tube itself or by the actual physical action of fixation. Each centrifuge tube used in the present experiments was approximately the same size and shape and had been treated by a similar cleaning process; it is difficult to imagine that there could be any difference between the tubes sufficient to cause such variation as is seen with specimen A (Table 1) where the percentage impregnation varied from a minimum of 62 per cent. to a maximum of 85 per cent.—a difference of 23 per cent.! It is always possible, however, that the process of impregnation is so delicate that the slightest trace of some impurity may upset it, as it is well known that in many chemical and physical reactions a trace of some impurity can act as a retarding agent which inhibits the normal working of the process (Bailey, 1937). It is always evident that the actual process of fixation can never be *identical* since a personal factor is always introduced, and it is possible that the speed and uniformity of the introduction of the fixative into the centrifuge tube plays a part in the determination of the maximum percentage impregnation. Since the controlling factor is introduced at the time of fixation, there is no evidence to suggest that with a given specimen of osmium tetroxide the later stages in the osmication play any part in the determination of the final impregnation figure. The fact that different specimens of osmium tetroxide give such widely different figures is further evidence that the impregnation process itself is a very delicate one that is easily inhibited by impurities; indeed it is difficult to account for the results of different samples by any other hypothesis. There is no evidence to indicate the nature of these impurities, but it is well known that the pH is an important factor in the reduction of metals from solutions, and it is possible that the impurities in different samples are such as to affect this factor.

The results of experiments with the solutions in constant rotation during fixation and osmication indicate that there is nothing to be gained by this technique which only produces extremely variable results. In some individual cases these are higher than those of normal fixation, but on the whole give very poor impregnation. It is difficult to account for the irregular results produced by the mixing, and there is no evidence to indicate what factor is thereby introduced. Considering the small size of *Chilomonas paramecium*, it is not surprising to find that the length of fixation time has no effect in determining the impregnation percentage; but it is interesting to note that the power of impregnation with a poor specimen of the osmium tetroxide is not increased by prolonging the fixation time, as was first thought might be the case.

MacLennan (1940), from work on a number of Protozoa, stated that the irregularity of impregnation was due to a variation in the osmiophily of the vacuolar region with the phase of the contractile vacuole, and that 'impregnation is consistent, *when like functional stages are compared*' (the italics are MacLennan's). Thus he considered his results to be uniform, and correlated his findings with the fact that, as opposed to metazoan tissues, we are dealing with organisms so small that each part is in close touch with the fluids concerned in the technique. He writes:

> 'In the Protozoa, the individual cells are separate and the distance of any granule from the free surface is measured in microns rather than millimeters. Thus, fixation, washing, impregnation and every other stage of the Golgi techniques are uniform with respect to every protozoan in the lot, rather than uniform only with respect to a narrow layer of cells equi-distant from the surface. Because of these advantages which are inherent in the Protozoa, it is possible to achieve uniformity of conditions both with respect to conditions of fixation and impregnation and also with respect to conditions within the cell. With these uniform conditions the Golgi impregnations give uniform results.'

These conclusions are open to serious criticism on the grounds that, as far as can be inferred from the data given in his paper, they are based on the results of a *single* series of experiments. Moreover, he does not take into account irregularities which may arise due to the specimen of osmium tetroxide used. It has been shown in *Chilomonas* that this is a factor of major importance, and it is unlikely that with the Protozoa used by MacLennan the same fact does not apply. It seems more likely that the irregularity of his results is due either to the osmium tetroxide used or to some inhibitory agent rather than to a variation in the osmiophily during the vacuolar phases. It is impossible in *Chilomonas* that the osmiophily varies with the vacuolar phases, for although in the present experiments the maximum impregnation reached with normal osmication was only 85 per cent., in a previous paper (Gatenby and Smyth, 1940)—where a better sample of osmium tetroxide was available consistent impregnations of 99 per cent. were obtained. Since the vacuolar phase must have varied greatly in the large number of organisms used it is evident that in *Chilomonas* there is no relation between the percentage impregnation and the vacuolar phase.

SUMMARY

1. Examination of the contractile vacuole of *Chilomonas paramecium* during progressive impregnation by the Weigl osmic technique revealed that the so-called 'osmiophil cortex' appeared first as a number of perivacuolar osmiophilic granules. Prolonged impregnation caused these to fuse to form a closed ring which, after very prolonged incubation, became a solid osmiophilic mass.

2. Cultures fixed together and osmicated in separate tubes gave more consistent impregnations, in any one series, than those both fixed and osmicated separately.

3. With the two samples of osmium tetroxide used, after normal fixation, A consistently gave impregnations of 62–85 per cent., and C impregnations of 25–36 per cent.

4. Constant mixing during fixation and osmication was not advantageous, and gave more irregular results than normal methods.

5. Time of fixation, between the limits of 5 to 90 minutes, had no effect on the impregnation.

6. It was suggested that the irregularities of impregnation with a given sample of osmium tetroxide were due to the presence of a trace of some retarding agent, possibly introduced at the time of fixation.

7. It was shown that the osmiophily of the perivacuolar region in *Chilomonas* did not vary with the vacuolar phase.

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On the Structure, Division, and Systematic Position of *Trichomonas vaginalis* Donné, with a Note on its Methods of Feeding

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With one Plate

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I. INTRODUCTION

TRICHOMONAS VAGINALIS, parasitic in the human vagina, is the type species of its genus. It is the largest, commonest, and most easily observed of the human trichomonads, and may be easily cultivated in media in ordinary use. Further, it is probably responsible, it now seems, for the vaginitis with which it has long been associated (Trussell, 1940; Trussell and Plass, 1940; Hogue, 1943). Obviously, *T. vaginalis* is an organism of some importance to the protozoologist and the physician; yet its life-history is imperfectly known and its structure and systematic position are still in dispute.

In 1926 Wenyon suggested that the characters then believed to separate the trichomonad of the human vagina from *Trichomonas hominis* Davaine, of the human intestine, might be due to the differences in their habitats. Were this so, and *T. vaginalis* and *T. hominis* synonyms, there was an evident possibility that infection of the vagina might be initiated by the migration of flagellates along the perineum from the anus. In the last 20 years, both experimental and morphological studies of the problem have led to disagreement. The examination of abundant material of *T. vaginalis* has convinced me that this is a good species, separable from all other human flagellates on purely morphological grounds. It is the main purpose of this paper to describe the evidence for this contention.

Andrews (1929) found that *T. vaginalis* in the vaginal secretion was a larger flagellate than *T. hominis* and had a shorter undulating membrane, the axoneme of which was not continued posteriorly into a free flagellum (Fig. 1); *T. hominis* had a long membrane with a free posterior flagellum. But these differences disappeared in culture and *T. vaginalis* assumed the form and dimensions of *T. hominis*. At that time the existing accounts of the structure of *T. vaginalis* by Künstler (1884), Blockmann (1884), Bensen (1910), Reuling (1921), Hegner (1925), and Schmid and Kamniker (1926) had left so much in doubt that Andrews's discovery was not sufficient ground for uniting the species and she did not formally propose to do so. But in 1934 Dobell isolated in culture a strain of *Trichomonas* from the gut of *Macacus nemestrinus*, passed it through the gut of *M. rhesus*, infected a human intestine with it, and, recovering it from the faeces, infected both the vagina and the gut of *M. sinicus*. Meanwhile, Bishop (1931) had given a clear account of the structure and division of *T. hominis* and Dobell referred to his flagellates as indistinguishable from those described by Bishop. He provisionally concluded that the intestinal and vaginal trichomonads *of man* belonged to the same species. This was disputed by Westphal (1935) and Powell (1936), who maintained that both forms were always distinguishable in culture. Karnaky (1934) claimed, on very slight evidence, to have infected the human vagina with intestinal trichomonads, but Stabler, Feo, and Rakoff (1941), Feo, Rakoff, and Stabler (1941), and Stabler and Feo (1941, 1942) failed to do so. Since 1926 new descriptions of *T. vaginalis* have been published by Wenrich (1931, 1939), Bland, Wenrich, and Goldstein (1931), and by Powell (1936). They all considered *T. vaginalis* distinct from any intestinal flagellate, but their argument was marred by disagreements about characters so fundamental in systematics as the structure of the nucleus and many cytoplasmic organs. (The American protozoologists in general recognize various subdivisions of *T. hominis* not universally accepted. This taxonomic treatment makes no difference to the position of *T. vaginalis*, but is based on distinctions which have never been urged for or against uniting *T. vaginalis* with any intestinal flagellates. Throughout this paper *T. hominis* is used in its wide sense to include the intestinal trichomonads of man and some monkeys (see Dobell, 1934).)

Clearly, we cannot assess the systematic position of *T. vaginalis* until the contradictory statements about its structure are resolved and we know enough about its mode of division to support comparison with that of other trichomonads. The following studies have been made to these ends. In addition, plentiful opportunities to observe the living parasites have enabled me to incorporate in this report some notes on their feeding methods.

II. MATERIAL AND METHODS

1. *Material*

All the flagellates used in this work were obtained from patients at the Exeter Women's Welfare Clinic. A little of the exudate was scooped from the vagina in a small spoon, a drop of it diluted with Ringer or the liquid component of the culture medium, and examined for parasites. Material was often available for study within a few minutes of leaving the host, but this is unimportant. Some strains remained active and normal for several hours when left at room temperature and I was never able to observe changes in any flagellate so kept for half an hour; material was almost always examined more promptly than this.

2. *Culture Methods*

I have attempted no systematic investigation of the reactions of *T. vaginalis* to different culture media, but have been concerned merely to find a method by which healthy stocks of flagellates could be maintained for use in the laboratory.

All cultures have been grown on variants of Boeck and Drbohlav's media as described by Dobell and Laidlaw (1926). After preliminary trials it was found that the medium known as Ere (coagulated egg slopes covered with diluted egg albumen) was easy to prepare and keep, and, after modification as described below, gave excellent cultures. Andrews (1929) found it unsatisfactory as excessive bacterial growth necessitated daily sub-culture; this may be remedied by the addition of a few drops of acriflavine (1 : 1000) to tubes containing 4–5 c.c. of liquid.

For some strains at least, additional carbohydrate is not essential for maintaining cultures, though it very greatly improves them. Without it growth may be good for a short time, but the flagellates eventually become extremely small, sluggish, distorted, and few; they recover a little on sub-culture but degenerate again within 24 hours. I have kept some strains in this condition for over 2 months, by sub-culturing every other day. The effect of adding carbohydrate to such stocks was startling. Trussell and Johnson (1941) noted great increases in the populations of cultures after adding glucose, maltose, soluble starch, dextrin, or glycogen to their medium, but, with Ere, this is not the only effect. After passing a strain through 25 sub-cultures in 60 days in ordinary Ere, I transferred it to Ere plus 0·25 per cent. dextrose (= Ere+d). (The substitution of soluble for solid carbohydrate reverts to the original recommendation of Boeck and Drbohlav, 1925.) At the time, prolonged search of three dippings from the old medium revealed two tiny, distorted flagellates, feebly beating their flagella from time to time; the strain had been in this condition for about 6 weeks. After 2 days on the new medium, a glance down the low power of the microscope showed in the first drop examined scores of large, vigorous, and normal trichomonads all over the field. In the old medium, measurements made over the 18 days before the transfer showed a few flagellates 8–9 μ, but most 5–6 μ in length, excluding the axostyle. After 24 hours incubation in the new medium, the first 25 brought at random under the $\frac{1}{12}$ in. objective varied from 10 to 18 μ, and after 48 hours all sizes from 8 to 24 μ, with a mean of 14 μ, were found in 50 measurements. The small individuals were presumably the products of the very rapid rate of division. Over the next 2 months on the new medium, tubes usually lasted for 6–7 days or even, though exceptionally, for a fortnight without sub-culturing.

The addition of sterile rice starch to culture tubes may have a similar invigorating effect, but it suffers from some disadvantages. Starch is difficult to sterilize. The grains, if ingested, tend to distort the bodies of the trichomonads. Some strains do not ingest starch grains at all; but it is of the greatest importance (see below) to understand that such strains may benefit considerably from the addition of starch to their medium; they increase in size, numbers, and mobility, though not to the extent of those fed on dextrose. One fact, important in diagnosis, remains to be noted. According to Dobell (1934), who cultivated *T. hominis* on all varieties of Boeck and Drbohlav's medium, primary cultures from faeces almost always showed abundant growths after 24 hours. *T. vaginalis* is less reliable. Primary cultures, with or without additional carbohydrate, were often feeble and short-lived. Flourishing sub-cultures have been obtained from primaries in which no trichomonads could be found except by prolonged searches of three or four dippings, i.e. from cultures very likely to be passed as negative when examined by quick routine methods. Early sub-culture from the primary tube is essential if negative results are to be accepted.

To summarize, the following method is recommended to give active, healthy, and prolific cultures of *T. vaginalis*. Slopes of whole egg are autoclaved and covered with 4–5 c.c. of a liquid consisting of the whites of 2–4 eggs in a litre of Ringer's solution plus 2·5 gm. of dextrose, sterilized by filtration. Incubate overnight to test for sterility; the medium is then approximately neutral. Inoculate with several globules of vaginal exudate held in a sterile platinum loop and incubate at 37°. Sub-culture next day and thereafter every 4 or 5 days according to the state of the cultures. If bacterial growth becomes excessive, add 3 or 4 drops of acriflavine to each tube for several generations. Trichomonads are found in the whitish pulp at the bottoms of the tubes where the solid component of the medium is breaking down. Sub-cultures are made by transferring from 0·5 to 1·0 c.c. of this in a sterile pipette—not a loop scraping from the slope—to a new tube.

3. *Cytological Methods*

Most authors agree that *T. vaginalis* is difficult to fix and stain satisfactorily, and I have found the reagents commonly employed to preserve cytological detail far more useful than the ordinary protozoological fixatives. Bouin's fluid caused so much distortion of the cytoplasm that it was almost useless. Alcoholic modifications of Bouin were very little, if any, improvement on the original aqueous formula, but Schaudinn's fluid with acetic acid and Dobell's modification of Zenker's fluid (Dobell, 1943) gave better results. The vapour of osmium tetroxide was useful for cytoplasmic fibrillae and flagella, provided that the smear was kept always moist. Other authors (Schmid and Kamniker, 1926; Westphal, 1935; Wenrich, 1939) have made use of dried smears, and by their methods I have produced preparations resembling some of their illustrations. After comparing these with wet smears, I am convinced that the damage done in drying is a dangerous source of error, all the more misleading because a dried film can often be stained more easily than a wet one. Most of this work is based on smears fixed for 15–30 minutes in modifications of Flemming's or Champy's fluids. The variants most commonly used were: (1) Flemming—1 per cent. chromic acid, 16 parts; 2 per cent. osmium tetroxide, 4 parts; glacial acetic acid, 1 part; and (2) Champy—3 per cent. potassium dichromate, 2 parts; 1 per cent. chromic acid, 2 parts; 2 per cent. osmium tetroxide, 1 part. (Unless otherwise stated, the terms 'Flemming' and 'Champy' in this paper refer to these modifications and not the original formulae.) Flemming gave the clearer general picture, including that of the nucleus, and Champy beautifully preserved the cytoplasm. Feulgen's 'nucleal' reaction has been tried; after fixation in Flemming or osmic vapour and hydrolysis in weak hydrochloric acid for 15–20 minutes or 7 minutes respectively, and staining for 3–4 hours, some positive results were obtained, but as an aid to morphological study the method was useless. I believe that the fault lies in the material rather than the technique, for I have obtained better results with similarly treated *T. muris*. The standard method of hardening fixed material in 96 per cent. alcohol for 48 hours, mordanting all day in iron alum and staining all night in Heidenhain's haematoxylin (both in aqueous solution), has given by far the most useful results. In addition, cytoplasmic inclusions have been studied with the aid of Janus Green and the Champy-Kull and Benda techniques for mitochondria.

It is necessary to say a word in explanation of the style of illustration chosen. *T. vaginalis* is a tiny, delicate, and complex organism, admittedly difficult to fix and stain. It has often been found impossible to judge from the more or less diagrammatic black and white pictures of some authors whether their confident assertions about minute and crowded structures were supported by their preparations. I have thought it best to supply portraits of individual flagellates as faithful as I could make them and leave the reader to assess their value as evidence for both my conclusions and my doubts.

III. GENERAL OBSERVATIONS AND FEEDING METHODS

1. *The Appearance of the Living Organism*

T. vaginalis is already sufficiently well known to require no lengthy general description. In length it varies from 6 to 30 μ exclusive of the projecting portion of the axostyle and when measured in its natural position as it lies freely in liquid. If correctly fed its size is maintained in culture. When undistorted by pressure, it is a plump, spindle-shaped flagellate, with four anterior flagella, a short undulating membrane, and an axostyle which often projects posteriorly (Pl. 1, Figs. 1, 2, and 5). Its cytoplasm contains numerous round, refringent granules which have been the subject of comment ever since Künstler, in 1884, mistook them for ingested bacteria. Almost every author notes that they tend to be arranged in several rows about the axostyle and the basal fibre of the undulating membrane. Now *T. vaginalis* is, as noted by Donné (1836), an exceedingly active and highly polymorphic organism, constantly stretching, contracting, and bending almost double, with strong flexures of the axostyle (Pl. 1, Figs. 2 and 3). These contortions imply that its cytoplasm is usually in a state of violent agitation, in the midst of which the axostyle and other fibres offer situations of relative stability about which the granules may repose. Granules remote from these skeletal structures are irregularly arranged and their agitation as the animal moves can be observed; the regular arrangement about the fibres is probably a simple result of the dynamics of their environment.

Twice in a vaginal discharge and frequently in culture, exceptionally large forms have been seen with plump, immobile bodies and a small, clear, mobile anterior region bearing the flagella and membrane. The largest and least normal of them exhibited long immobile lobes or 'fingers' of hyaline cytoplasm and were degenerating. According to Hogue (1944), who made a special study of these forms, they have the peculiar habit of autotomizing the large posterior region. She considered them to be produced by the physical properties of the semi-solid medium she employed, but I have invariably found numerous smaller, normal trichomonads accompanying the abnormal types. From the fact that they occurred only in exceptionally heavy infections or in culture tubes with abnormally high populations, I am inclined to regard them as hypertrophied individuals produced by overfeeding in a specially nutritious environment.

2. *The Feeding Methods*

Setting aside the erroneous views of Künstler (1884), who mistook the axostyle for an oesophageal tube, we find that later authors give the impression of great uncertainty about the cytostome and feeding (Reuling, 1921; Hegner, 1925; Wenrich, 1931, 1935, 1939). Schmid and Kamniker (1926) thought that a cleft at the anterior end of the body between their two blepharoplasts might have been a cytostome. They put forward the unusual view that the flagella were withdrawn into this cleft and protruded, on stimulation by foreign bodies, to fish for food; leucocytes were supposed to be ingested by pseudopodia. Their observations on living material were supported by some on flagellates fixed by drying and stained as Gram smears which cannot be accepted as evidence for their isolated opinions. Bland *et al.* (1931) included a cytostome in their diagram of *T. vaginalis*, but omitted it without comment from their paper of the following year. All these authors (except Schmid and

Kamniker) agreed that food inclusions were rarely seen in the cytoplasm and Reuling thought that feeding might be osmotic. Bland *et al.* (1932) found little evidence that *T. vaginalis* ingested bacteria in nature, though they did so in culture. Powell (1936) believed that there was no cytostome and supported his views with a clear description of feeding, with most of which, as far as it goes, I am in agreement. *T. hominis* has a cytostome through which it feeds on small bacteria (Dobell and O'Connor, 1921).

T. vaginalis feeds on small particles such as bacteria and the detritus derived from broken-down coagulated egg and on larger objects such as erythrocytes and starch granules and probably on food in solution. Three main methods can be distinguished. (i) When feeding on bacteria and other minute particles in the liquid part of its environment, *T. vaginalis* usually attaches itself by its axostyle to a cell or other firm base with its body leaning out into the liquid, constantly extending and contracting and twisting, with flagella and undulating membrane incessantly working. There is no regular food stream directed to any constant point on the body. The flagella do not beat one after the other in series but all together, like the thongs of a knout, usually away from the undulating membrane, but sometimes towards it. By their action, bacteria are knocked against the body, usually posteriorly; some are carried there by the membrane, but this does not seem to set up any well-marked food current or to have any special importance in feeding. Most, if not all, of the particles slip off, but some are recovered by the flagella which knock them back again and again on to the body and, sooner or later, one of them adheres to the surface. It is noticeable that when a particular bacterium is receiving attention, the apparently accidental blows of the flagella may be supplemented by stretching and bending movements of the body as if the flagellate were reaching out for the particle. Eventually small groups of bacteria and other particles make tiny mounds attached to the body surface. Very slowly, over periods of an hour and more, they disappear. I have never seen any pseudopodial action during this process, which is so gradual that it is impossible to say, from direct observation, whether disappearance of the particles indicates that ingestion has taken place, but minute inclusions, though rare, are sometimes seen in the bodies of *T. vaginalis* and some particles must at times be ingested, though probably not as a general rule. (ii) Large objects, such as starch grains, are dealt with by a method which is a modification of that already described, but in which the flagellate makes far greater use of its body to hold the object. It wraps itself round as much of the grain as possible, at the same time clasping it with its flagella, which move only slightly unless the grip is lost, when they knock it back into position with repeated blows. From time to time the flagellate may shift by relaxing its hold and sliding round the grain to attack it from a new angle. After prolonging this treatment for over an hour, trichomonads have been seen, on straightening out, to have ingested small starch grains which distorted their cytoplasm. (iii) *T. vaginalis* burrows deeply into the soft debris found in the bottom of culture tubes (Bland *et al.*, 1932).

Individuals have frequently been seen, quite motionless, and partly hidden in semi-solid accumulations of egg debris; if some movement of the surrounding liquid disturbed them, it was noted when they emerged that granules of food material were adhering to them; they had evidently been feeding without the use of their flagella. At no stage in any of these processes have I ever seen a cytostome in use, nor can I identify one with certainty in fixed preparations.

The following facts appear to support the view that extracellular digestion is common among these flagellates. Food inclusions have very rarely been seen, and this is in agreement with the observations of most other workers; yet all flagellates capture bacteria and, indeed, spend almost all their time doing so; no strains ignore them. (Bland *et al.*, 1932, held that adherent bacteria were symptoms of 'lowered vitality', but they offered no supporting evidence and the universality of the phenomenon among even my healthiest stocks argues against their supposition.) Strains which do not ingest starch grains pay attention to them in the way described. This might be attributed to the exercise of a common reaction, however unsuccessful, to food material or to solid particles of the right size; but, as already explained, the addition of starch improves cultures of strains which do not ingest it. Similarly, human erythrocytes were treated like starch grains by strains which did not ingest them and, on being discarded, they were seen to be distorted in outline although untouched corpuscles on the same slide retained their normal appearance. Finally, as Westphal (1935) pointed out, the adherence of particles is not due to the general stickiness of the body surface; it is the symptom of a localized, special, physiological condition such as would be produced by a secretion at the point of adherence.

IV. Structure and Division

1. *Cytological Structure*

In stained preparations, the most conspicuous feature of *T. vaginalis* is usually the large number of granules already mentioned (Fig. 3). They vary slightly in size and more in number and distribution. They fill up much space and obscure most of the other cytoplasmic structures as well as the nucleus. Unfortunately, they are best preserved by those fixatives which least distort the rest of the body. After fixation with Champy or Flemming without acetic (original and modified formulae), they are spherical and stain very deeply with iron haematoxylin. Acetic acid does not completely destroy them, but commonly attacks them so that, after Bouin or Schaudinn fixation, they are less clear-cut and stain less deeply, appearing sometimes as obscure dark blots; in such preparations, where preservation of the granules is imperfect, there is a constant danger that they may be mistaken for food inclusions, and when, as very frequently, they surround the nucleus, it is most difficult to distinguish the outline and contents of that organ; conclusions based on methods likely to be affected by this source of error should be considered with great caution. Champy-Kull preparations, with or without post-chroming, showed the granules brilliantly stained with acid fuchsin. After staining by Benda's alizarin crystal-violet aniline water method, the granules were violet. Apart from the difference in colour, the appearance of the granules after these last two treatments is that shown in Fig. 3. Although these facts were consistent with the view that the granules were mitochondria, it was never possible to stain them vitally with Janus Green (1 in 10,000 for upwards of an hour).

The 'resting' nucleus is oval, with a delicate membrane, the outline of which is not reinforced by any marginal concentration of chromatic material. There is a single karyosome, usually surrounded by a slight halo (Figs. 1, 1 A, and 2). (The term 'karyosome' is here used descriptively as applied to similar nuclear granules in other trichomonads and without implying more about its homology and nature than stated in this paper.) The rest of the nucleus stains only faintly and is probably structureless; a slight unevenness in its consistency is most probably an artifact. Neither karyosome nor membrane stains strongly after Champy; both are more evident after Flemming. I have never found any trace of the karyosome in Feulgen preparations in which dividing nuclei were stained and I believe the 'resting' phase to be Feulgen negative. This stage is relatively uncommon, most of the nuclei being in one of the phases preparatory to division; for this reason, and because there is little to stain in the nucleus, it is easily overlooked.

Immediately before the nucleus, at the anterior tip of the animal, lies the blepharoplast. It is a deeply staining, compound structure, usually lobed so as to suggest, especially in almost completely destained specimens, that it consists of four granules overlapping each other. During the early stages of division, when the constituent granules separate a little, the four can be distinctly counted (Figs. 4 and 7), but whether they remain as discrete entities throughout the trophic phase I have been unable to determine; they were too tightly packed. From the blepharoplast, the flagella, undulating membrane and its basal fibre, the axostyle and the parabasal fibre take their origin. According to Powell (1936), the blepharoplast always consists of five granules to which these motor and skeletal elements, as well as a rhizoplast, are all related in a 'very definite and constant fashion'. After many attempts to follow him, I find it impossible to make any such positive statement about the arrangement of these tiny and crowded objects. I believe that ordinarily the axostyle is attached to a granule on one side of the blepharoplast, and, on the other side, the axoneme of the undulating membrane and its fibre to a second, and the parabasal fibre to a third granule (Fig. 4); all the flagella are not attached to the same granule, but I am uncertain to which they belong. I am unable to find the rhizoplast or fifth blepharoplastic granule. This description of the relations of the blepharoplast to its fibres, so far as it goes, has been checked frequently in trophic organisms and is consistent with the behaviour of the granules and organellae during division; no evidence against it has been observed.

The four flagella are usually at least as long as the body of a fully grown trichomonad and their length is fairly constant, so that they seem disproportionately long in small animals. They often appear to be proximally joined in pairs for a short distance. I can find no support for Andrews's contention that one pair is slightly longer than the other. I have occasionally come across specimens of *T. vaginalis* which seemed to have only three flagella; these were in smears fixed in Champy or Flemming, not in living material or osmic fixed smears—which were very reliable for flagella counts—and I attach little importance to them.

The undulating membrane, as all recent authors agree, is short and confined to the anterior half of the body, and its axoneme is not continued posteriorly as a free flagellum. This I have found to be true of all fresh material and, with two exceptions, of cultivated trichomonads. The exceptions were both primary cultures, in Ere, with, in one case, the addition of 0·5 per cent. sodium citrate to the liquid component of the medium; the populations were low and the flagellates rather small. The first sign of abnormality was noted on the fourth and fifth days respectively; in most of the flagellates the membrane was exceptionally long. On the following day it had grown right round the body as far as the axostyle and a few flagellates had a long, trailing flagellum leaving the body at the end of the membrane and hanging limply beside the posterior, protruded length of the axostyle. Next day nearly all the flagellates were in this condition and the trailing flagellum was about as long as the body; it was maintained in sub-culture. I had not at that time discovered the use of dextrose and was unable to produce flourishing cultures from which preparations could be made. The history of these two strains seems to be similar to that of Andrews', except that in mine the flagellum was a little slower in developing. Forty-six other strains kept for periods of 8 days to 5 months and examined for this point failed to show any significant variation in the length of the membrane. Nor was anything resembling a *Eutrichomastix* phase ever seen; probably the shortness of the membrane in *T. vaginalis* explains the absence of those accidentally produced forms—common enough in trichomonads like *T. muris* with long, powerful membranes—in which the axoneme is torn away from the body right up to the blepharoplast, thus imitating the structure of *Eutrichomastix*.

A slender, deeply staining basal fibre—shorter and much more delicate than the long, thick rod which is so prominent a feature of *T. hominis* preparations—leaves the blepharoplast and runs below the undulating membrane (Figs. 1, 2, 4, and 5). It is a skeletal structure; in both living and fixed material individuals have been seen in which the fibre was pulled out from its normal position and, hinging on the blepharoplast and carrying the membrane with it, stretched the cytoplasm between it and the axostyle as the skeleton of a bird's wing stretches the patagial membrane.

The flexible axostyle leaves the blepharoplast to curve round the nucleus on the opposite side to the undulating membrane, and then, if at rest, usually straightens out to run directly to the posterior end of the organism from which it may protrude (Fig. 1). Variations in its position may be seen in Figs. 2, 3, and 5, where, if the whole extent of the axostyle is not visible, its course is indicated by the granules around it. In small flagellates reared without additional carbohydrate, the exposed posterior portion was relatively long. The axostyle is a slender, siderophile structure, only very slightly broader anteriorly than posteriorly and of about the same width and appearance, except for length, as the basal fibre; it was always very easily distinguishable from the thick, tapering rod, with siderophile edges and hyaline core, found in such forms as *T. hominis* and *T. muris*.

A third siderophile fibre, of variable length, arises from the blepharoplast and curves round one side of the nucleus, between it and the basal fibre, towards the axostyle. Below the nucleus it may cross the axostyle and continue for some way beyond it before tapering to an end (Figs. 1, 1 A, 2, and 5); sometimes it fails to reach as far as the axostyle and occasionally it ends at its point of contact with that organ, so as to give the false impression —apparently that which misled Reuling (1921)—that it forms one side of an axostyle anteriorly expanded to embrace the nucleus. Like the basal fibre and the axostyle, it is flexible and is variously curved by the movements of the body. This delicate, deeply staining structure, which often resembles the basal fibre so closely as to suggest a duplication of it, is the parabasal fibre. There is no difficulty about its demonstration and no dispute as to its existence; it has never been described in *T. hominis*. The same certainty does not apply to the parabasal body of *T. vaginalis*, the presence of which has been affirmed, denied, or ignored by different authors (see p. 94). After fixation in Flemming or Champy, prolonged staining in iron haematoxylin (24 hours each in mordant and dye), and appropriate differentiation, the parabasal body is stained grey, and this is the most reliable method of demonstrating it. In Benda preparations it is often visible, stained purple, and it may also be stained by leaving smears overnight in Delafield's haematoxylin, diluted with distilled water to about one-twentieth of its normal strength. After fixation in Schaudinn, the body is very rarely demonstrable with any degree of certainty. The staining reactions of the parabasal body are admittedly somewhat variable and, in smears differentiated to show nuclear detail, it is often completely decolorized in every specimen; except in dried smears, which I distrust, I have never seen it so sharply defined as it appears in some of the illustrations published by Wenrich and his collaborators (Wenrich, 1931, 1939; Bland *et al.*, 1931). But, in spite of its apparent absence in some smears, and some uncertainty about its identification in others, it is recognizable in too many individuals, especially after the first treatment described above, for it to be dismissed as an artifact. In appearance the parabasal body is a band of lightly staining material of rather uneven texture, adhering to the parabasal fibre, along which it stretches for a variable length, rarely less than half-way from the blepharoplast and usually not quite to the tip. It is ordinarily continuous (Figs. 1 and 2), but sometimes broken up into separate patches of stainable material (Fig. 1 A). Its most common appearance is that of some thick, coagulated, semifluid substance accumulated about the parabasal fibre and present in varying quantities in different individuals. I have occasionally found specimens with two parabasal bodies, each related to its own fibre. Wenrich has suggested that such individuals are preparing for division, but to this I cannot assent. Normal dividing forms possess only a single parabasal fibre up to a late stage in the process, when a second fibre arises *de novo*. The forms figured by Wenrich and those seen by me are more likely cases of abnormal duplication; doubling of various structures is a fairly common abnormality of this and other trichomonads (see below, and Bishop, 1931).

2. *Division*

Very little about the division of *T. vaginalis* is to be found in the literature. It will be understood that, although dividing stages are common enough in heavy infections and good cultures, few of these are of any use to the investigator. The great majority have their nuclei and other structures obscured by the granules already mentioned. Also, it is usually impossible so to differentiate stained preparations that the conditions of the nucleus and cytoplasmic organs are accurately revealed in the same specimen, e.g. the flagellates shown in Figs. 7–9 accurately display the nucleus, but are unreliable for fibrillae, while that in Fig. 17 shows the reverse state.

The first indication that the flagellate will divide is the appearance within the nucleus of chromatic granules outside the karyosome. They increase in number and the nucleus elongates. For a while the faintly staining karyosome is still discernible (Figs. 4 and 5), but it soon disappears. The nucleus now reacts positively to the Feulgen technique (Fig. 6), and continues to do so throughout division. It is very difficult to count the granules accurately, but there are about twenty. At about the same time the tight complex of blepharoplastic granules is slightly relaxed (Figs. 4 and 7), but, typically, it is the nucleus which shows the first signs of division. These changes result in the appearance most commonly found in stained smears and frequently figured in the literature, though not previously recognized for what it is; I conclude that the phase is prolonged. The body of the dividing trichomonad now usually rounds off, but occasionally an elongated, more or less oval form is retained up to the time of cytoplasmic fission. The following stages are rarely seen and presumably take place rapidly. The nuclear membrane disappears, but the chromatic contents of the nucleus lie in a clear, pale area distinct from the surrounding cytoplasm. The numerous small granules begin to associate in groups, at first rather indefinitely (Fig. 7), but in the end five small aggregates are found, in which the granules are at first plainly separate but finally begin to lose their identity (Figs. 8 and 9); in this way five more or less ellipsoidal chromosomes, all about the same size, are formed (Fig. 11). My opinion is based on the small number of individuals in which the nuclear structure during these stages was perfectly clear; the chromosomes, when formed, usually overlapped, but in a few cases no confusion was possible and then five chromosomes could be distinctly counted (Fig. 11); views of earlier and later stages in division were consistent with the opinion that five is the correct number of chromosomes. Meanwhile, a dynamic and rapid reorganization of the cytoplasmic structures takes place in a way difficult to follow, as the fibrillae lose their distinctive characters; always very similar in appearance, they stain only weakly in the earlier stages of division, and, at the same time, they shorten, except for the parabasal fibre, which seems to retain its normal length and is thus usually the longest of the fibrillae at this stage. Division of the blepharoplast takes place by the dissociation of its constituent granules during prophase; one granule divides and its two daughters, the centrosomes, pass to opposite poles of the nucleus; they remain attached by a stout, deeply staining centrodesmus. Of the remaining granules, two remain associated with one centrosome and one goes with the other (Figs. 8 and 9). The cytoplasmic fibrillae accompany the granules to which they are attached, so that the shortened axostyle passes to one pole, while the undulating membrane and its basal fibre, with the parabasal fibre, go to the other (Fig. 10). The separation of these organellae follows the course expected from their arrangement about the 'resting nucleus'. There is no good reason to suppose that any of the fibrillae disappear at any time during division; their presence, though not their identity, is often faintly revealed in more deeply stained individuals than those portrayed in Figs. 8, 9, 11, and 12, and, when they again become recognizable, they are found to occupy the positions in which they were last plainly visible (Fig. 14). The flagella are divided between the daughter blepharoplasts at the same time as the fibrillae, but I am unable to explain precisely how they are disposed.

I have not seen any arrangement of the chromosomes which constituted a typical metaphase; there is no spindle or equatorial plate, and, perhaps correlated with their absence, the chromosomes do not divide simultaneously, but irregularly; they apparently split transversely (Figs. 12 and 13), their daughters passing rapidly to opposite poles, while the centrodesmus elongates (Fig. 15). The chromosomes very soon lose their individuality and the commonest telophase picture shows that they have disintegrated into granules like those seen in early prophase (Fig. 16). This is the last stage to give a positive Feulgen reaction. By this time each blepharoplast has three granules in addition to the centrosome (Fig. 16). The new fibrillae appear suddenly; they grow out *de novo* from the blepharoplast, and there is nothing to suggest that any of the old fibrillae divides. In smears in which the parabasal body was consistently stained, dividing forms showed that a single body was present at one pole towards the end of prophase (Fig. 10), while some telophase figures had one at each pole (Fig. 17); I have found no evidence that the parabasal body is self-perpetuating.

Division of the cytoplasm is longitudinal and is initiated by the appearance of a cleft between the two sets of organellae, so that the body of the parent is heart-shaped, with a set of organellae in each lobe of the heart. During this time the dividing organism is very active, with flagella and membranes working vigorously, feeds, and, like trophic forms, is highly polymorphic. At no stage could any sustained pull between the two daughters be observed; both seemed to behave as independently as their attachment allowed. Their movements gradually deepened the cleft until they were connected by only a slender thread. This finally snapped, apparently as the accidental result of some particularly violent movement of one or both daughters, and division was complete.

I am unable to offer any account of the fate of the centrodesmus. It is present up to the last moment before final separation of the daughter organisms. The appearance of forms such as those shown in Figs. 15 and 16 suggests that when the centrodesmus snaps each of its halves completes the number of fibrillae required by the new flagellate, and for a long time I sought confirmation of this view. Instead I have found forms like that in Fig. 17, which have a full complement of fibrillae, though some not yet fully grown, at each pole, as well as a centrodesmus. Presumably, therefore, the latter is resorbed, as in *Trichomonas caviae* Dav. (Grassé and Faure, 1939), though it seems probable that the centrosomes are incorporated into the blepharoplasts as fourth granules (Fig. 16).

Various abnormalities, presumably the results of irregular fission, occur in *T. vaginalis* as in other members of its genus. Of these the commonest are (*a*) flagellates with two sets of organellae, including the nucleus, (*b*) those with two or even three additional nuclei, usually in the middle or posterior part of the body, but with only a single set of cytoplasmic organellae. Dislocation of the synchrony between nuclear and cytoplasmic division leads to occasional forms in which the nucleus reaches the end of prophase without any sign of division of the blepharoplast. Abnormal nuclei, containing a confused mass of chromatic blocks, clumped closely together, or with numerous large round granules in mulberry formation, also occur: I can offer no explanation of them. I have never observed in *T. vaginalis* the curious multiple forms illustrated by Bishop (1931, Fig. 60).

V. Discussion

In separating the species of human trichomonads, previous authors have made use of differences in size and such characters as the structure of the nucleus, axostyle, undulating membrane, parabasal apparatus, and other cytoplasmic inclusions. In regard to most of these points there is surprisingly little agreement.

Size. T. vaginalis is the largest trichomonad found in man, with a length, excluding the axostyle, sometimes reaching 30 μ. The greatest length recorded for an intestinal trichomonad seems to be 20 μ for *T. hominis* (Dobell and O'Connor, 1921) and for '*Pentatrichomonas ardin-delteili*' (Wenrich, 1931; Powell, 1936). *T. tenax* is smaller, with maxima of 12 μ (Bland *et al.*, 1931) or 17 μ (Powell, l.c.). The large size of *T. vaginalis* may be maintained in culture and the fact that starved specimens may be reduced to the dimensions of *T. hominis* (Andrews, 1929) is not significant. I can find no evidence that there may be two size races of *T. vaginalis* (Bland *et al.*, 1931); even the authors themselves admitted that the size distinctions disappeared in culture.

Nucleus. The most serious defect in the existing accounts of *T. vaginalis* is lack of consistency in descriptions of the nucleus. It has a karyosome (Bensen, 1910; Reuling, 1921); it sometimes has one (Hegner, 1925; Wenrich, 1931); none could be found (Powell, 1936); Bland *et al.* (1932) figured four distinct types of nuclear structure for this species. Some misunderstandings have undoubtedly arisen from failure to recognize early stages in mitosis for what they were; others, e.g. references to a structureless nucleus, seem due to faulty technique or observation. In specimens with unobscured and clearly stained 'resting' nuclei, I find that a karyosome is always recognizable. *T. hominis* has a similar nucleus, though its karyosome and nuclear membrane are more conspicuous. The fine extra-karyosomatic granules seen rarely by Bishop (1931) but thought by Dobell and O'Connor (1921) to be an ordinary feature of the nucleus of *T. hominis* may well mark the earliest preparations for division in that species, as they do in *T. vaginalis.* However that may be, the 'resting' nuclei of both species are almost identical. The nucleus of *T. tenax*, containing several large chromatic masses, is easily distinguishable from that of *T. vaginalis* (Hinshaw, 1926).

Axostyle. According to Reuling (1921) the axostyle consists of two fibrillae which embrace the nucleus (probably the axostyle and parabasal fibre) and two more from the nucleus itself. Hegner (1925) saw the axostyle as a single, slender fibre, but thought he might have been examining the edge of what was really a thick, hyaline rod. He was possibly influenced by the fact that the latter is the commonest type of axostyle in trichomonads; it occurs in *T. hominis* (Dobell and O'Connor, 1921; Bishop, 1931) and has been attributed to *T. vaginalis* by Wenyon (1926), Andrews (1929), Powell (1936), and by Wenrich and his collaborators. In a later paper Wenrich (1939) asserted that the axostyle was composed of two to eight fibrillae, which, in abnormal individuals, became more or less separated and distinct; the phenomenon was said to be especially clear in dried films. I believe that the axostyle, as revealed in wet preparations, is a single, deeply staining fibre, similar to the basal fibre (Figs. 2 and 5). Its structure was correctly given by Westphal (1935), who realized that in this respect *T. vaginalis* differed from *T. hominis.* Hinshaw (1926) described the axostyle of *T. tenax* as a slender siderophile rod; it resembles that of *T. vaginalis.* It is worth noting that when the new axostyles of *T. hominis* first appear during division they are slender fibres (Bishop, 1931, Fig. 54) with an unmistakable resemblance to the new axostyles of *T. vaginalis* (Fig. 17); the stout, hyaline structure seems to be secondary.

Undulating membrane. All workers, except Künstler (1884), are agreed that the undulating membrane and its supporting basal fibre are normally short, extending about half-way or slightly less down the body from the blepharoplast. Since Andrews (1929) reported that in culture her specimens developed a long membrane and, from it, a free posterior flagellum, other investigators have occasionally seen the long membrane, but only Westphal (1935) found the posterior flagellum, and that but rarely. Two whole strains of mine grew a long membrane and posterior free flagellum, but I have never found such forms, as Westphal reported them, as exceptional individuals in otherwise normal populations. Bland *et al.* (1932) and Powell (1936) thought that the long membranes in such specimens of *T. vaginalis* belonged to truncated individuals which had autotomized their posterior ends. Against this explanation as a general rule may be argued (1) that autotomy is associated with degeneration (Hinshaw, 1926; Hogue, 1944) and healthy forms may have full-length membranes, and (2) that in my two strains, as in Andrews', the development of the long membrane was gradual and occurred in animals no smaller than those with normal membranes. It must be borne in mind that this phenomenon has never been seen in nature and, in my experience, is rare in culture. The occasional development of this similarity to *T. hominis* may indicate phylogenetic affinity, but is not a proof of specific identity any more than the resemblance between the membranes of *T. vaginalis* and *T. tenax* proves *their* identity. According to Westphal (1935) and Wenrich (1939), between the margin of the membrane and the axoneme a more delicate filament is to be found in dried preparations, but only rarely in wet ones. Similar filaments are said to be demonstrable by Bodian's protein-silver technique in '*Pentatrichomonas hominis*' (Kirby, 1945) and a trichomonad presumed to be *T. limacis* Dujardin (Kozloff, 1945). Basing my opinion on wet preparations, I am unable to depart from the conservative view that the axoneme forms the margin of the membrane in *T. vaginalis*.

Little is known about the *parabasal apparatus* of human trichomonads. There seems to be no doubt that, though Powell (1936) was unable to find the chromophobe body, it does occur in *T. vaginalis*, as Wenrich and his co-workers have consistently maintained. But their use of this structure in separating *T. vaginalis* from *T. hominis* is difficult to support. We know practically nothing about it in the latter species; Dobell (1942), in a paper on staining technique, incidentally comments that he has stained the parabasal body of *T. hominis*; Kirby (1945) has suggested that a small, ellipsoidal body lying near the nucleus, and which he impregnated with silver, might be a parabasal body. Even apart from these dubious circumstances it seems injudicious to employ in taxonomy a character demonstrable only with difficulty. The parabasal fibre is a different matter; it is easily seen in all ordinary preparations of *T. vaginalis* and has never been described in *T. hominis*.

The large cytoplasmic granules of *T. vaginalis* closely resemble mitochondria in their reactions to fixatives and stains. The addition of small quantities of acetic acid to fixatives did not destroy them, though it usually impaired their affinity for stains; it is already known that some mitochondria are resistant to weak concentrations of acetic acid (Nicholson, 1916). No technique has so far revealed any other inclusions which could possibly be interpreted as mitochondria. On the other hand, the granules failed to stain with Janus Green. Hogue (1922), relying exclusively on Janus Green, described the mitochondria of *T. hominis* as a few short, blunt rods. Whatever decision may eventually be reached about the granules in *T. vaginalis*, their treatment by Wenrich (1931, 1939) as specific characters seems open to the same objections as his similar treatment of the parabasal body.

Division. Apart from a few isolated sketches, the only previous account of the division of *T. vaginalis* is the admittedly incomplete one by Powell (1936). The earliest stages in *T. vaginalis* agree in general with those described by Bishop (1931) in trichomonads from the gut of man and *Macacus nemestrinus*. In all, as the karyosome disappears, chromatic material becomes recognizable outside it, but the large number of small granules in *T. vaginalis* contrasts sharply with the small number of larger granules in Bishop's material. As early prophase is prolonged this stage is commonly met with and the appearance of the nucleus is then characteristic of *T. vaginalis*. According to Powell, this species has four chromosomes. I am alive to the dangers of contradiction on such a point in this difficult material, but feel some confidence in correcting the figure to five. Wherever all the chromosomes could be clearly and separately observed there were five of them, and there were always five aggregates of granules in such stages as those in Figs. 8 and 9. Less convincing views of the nucleus in these stages, though not decisive, often supported my interpretation. Moreover, I have tried Powell's method of fixation with very hot Bouin and find it difficult to understand the deformations produced by it.

Complete demonstration of the relations of the blepharoplast has eluded me. It consists of four granules and one supplies the centrosomes by division. Partition of the parent's fibrillae to their poles and growth of the new basal fibre and undulating membrane take place somewhat earlier in *T. hominis* than in *T. vaginalis* and *T. tenax*. In all species the new axostyle is formed relatively late. There is no division of the old axostyle, but in *T. hominis* the old one is absorbed and two new ones grow out from the blepharoplasts. Hinshaw (1926) thought that this was true of *T. tenax* also, but he was admittedly swayed by evidence from other species, and his illustrations show the old axostyle still present at anaphase and perhaps even at telophase. I have never found convincing evidence that the old axostyle totally disappears during the division of *T. vaginalis* and believe that it persists, though shortened and with a greatly reduced affinity for stains. I have not discovered how the full complement of granules is restored to each daughter blepharoplast; some, at least, of the new flagella are, as in *T. tenax*, the last organs to appear, and it seems likely that they are related to the new granules.

The systematic status of the vaginal trichomonads of man. The work of Dobell (1934) mentioned on p. 80 provides very convincing evidence of the identity of the strains of *Trichomonas* he employed, but it is of great importance to note that these did not include any from the human vagina; the author himself suggested that the taxonomic question would only be settled by experiments on human beings. It is my contention that it has been settled by the disclosure of constant structural differences between *T. hominis* and *T. vaginalis* of sufficient significance to justify their separation as good species. When fully grown, *T. vaginalis* is larger than any other human trichomonad ever is. Its nuclear organization differs unmistakably from that of *T. tenax* and, if its life-history be pursued into prophase, just as clearly from that of *T. hominis*; numerous prophase nuclei are found in almost every smear and it should not be difficult to avoid confusion. The thick, long, heavily staining basal rod which is such a striking feature of *T. hominis* is replaced in *T. vaginalis* by a shorter and much more delicate, though well-defined, fibre. The fibrillar axostyle of *T. vaginalis* resembles that of *T. tenax* and is quite different from the stout, hyaline rod with siderophile edges found in *T. hominis*. The foregoing points are so constant and so easily demonstrable that they may be properly used in diagnosis. In addition, *T. vaginalis* differs from *T. hominis* in some ways which, either because they are imperfectly understood in one species or another or because they are difficult to observe, may be used only to augment a case for differentiation established independently; here might be included the structure of the parabasal apparatus, length of the undulating membrane, presence or absence of a free posterior flagellum, and nature of the cytoplasmic inclusions. It might, of course, still be maintained (*a*) that *T. vaginalis* and *T. hominis* may each survive in the typical habitat of the other, and (*b*) that if so they would then lose their specific characters. With regard to the former possibility, whatever view is taken of the evidence against it, no serious evidence in favour of it exists; the latter possibility is mere conjecture. In these circumstances, and taking into consideration the structural differences between these trichomonads, the onus of proof lies on any who still maintain that they are specifically identical.

In the course of this work I have had the benefit of some discussion with Dr. Ann Bishop, who also kindly allowed me to compare some of her preparations of *T. hominis* with my own material; it is a pleasure to acknowledge the help that this has been. My thanks are also due to Dr. Margaret Jackson and Mrs. L. A. Harvey for supplying me with material and to the latter also for much friendly help and advice; to Professor D. L. Mackinnon, who lent me much of the literature needed and read the draft of this paper; and to the authorities of this college for a grant towards the cost of the illustrations.

VI. SUMMARY

1. *Trichomonas vaginalis* has been cultivated on various media and a simple method of maintaining cultures on Boeck and Drbohlav's egg-Ringer-albumen medium is described.

2. The feeding methods of the flagellates in culture have been studied and some evidence of extracellular digestion is recorded.

3. When cultivated on media deficient in carbohydrate, *T. vaginalis* is reduced to the dimensions of *T. hominis*, but when adequately fed it main-

tains its distinctively larger size. Out of forty-eight strains of *T. vaginalis*, only two developed in culture the long undulating membrane and free posterior flagellum typical of *T. hominis* in nature.

4. The structure of *T. vaginalis* has been reinvestigated. (i) It differs constantly and significantly from the intestinal trichomonads of man in size, nuclear organization, and the form of the axostyle and basal fibre. (ii) It also differs from *T. hominis* in the characters of its parabasal apparatus and cytoplasmic inclusions, and, in nature, in the length of its undulating membrane and in lacking a free posterior flagellum, but for reasons discussed in the text these points are at present considered less reliable in diagnosis than those given under 4 (i).

5. It is concluded that *T. vaginalis* is a species distinct from all other human trichomonads.

6. The method of division has been described. In general, it follows the same course as *T. hominis*, but separation of the old, and growth of the new, cytoplasmic structures occurs somewhat later in *T. vaginalis*, and the old axostyle is retained throughout division. There are five chromosomes, formed during prophase from aggregations of extra-karyosomatic granules.

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EXPLANATION OF PLATE I

All figures are portraits of *Trichomonas vaginalis* made with the aid of a Leitz cam. lucida, and are ×2,350. Except where otherwise stated they were stained in Heidenhain's iron haematoxylin. Figs. 7–9 and 11–13 were differentiated to show nuclear detail and are unreliable guides to cytoplasmic structure; the reverse applies to Figs. 10 and 17.

Abbreviations: *Ch.*, Champy's fluid. *Feul.*, Feulgen's reaction. *Fl.*, Flemming's fluid. *Os.*, Osmic vapour.

Fig. 1. Trophic form with continuous parabasal body. *Fl.*

Fig. 1 A. Trophic form, anterior end only, with discontinuous parabasal body. *Fl.*

Fig. 2. Trophic form; note coagulated appearance of parabasal body. *Fl.*

Fig. 3. Trophic form, showing cytoplasmic granules. *Ch.*

Fig. 4. Anterior end of an individual preparing for division; extra-karyosomatic granules are appearing in the nucleus and the blepharoplastic components are dissociating. *Fl.*

Fig. 5. Slightly later stage than the last, with more numerous granules, and the karyosome disappearing. *Fl.*

Fig. 6. Prophase form showing only the nucleus, now without a karyosome. *Os. Feul.*

Fig. 7. Beginning of chromosome formation, with granules associating in indefinite groups. Note the blepharoplast. *Fl.*

Fig. 8. Five groups of granules recognizable and the centrosomes are disjoined. *Fl.*

Fig. 9. A slightly later stage than the last. Note centrosomes and blepharoplastic granules. Part of body omitted. *Fl.*

Fig. 10. Approximately the same stage as the last, more deeply stained. At one pole are the membrane and its shortened basal fibre, the parabasal body, and its now relatively long fibre; at the other pole is the shortened axostyle. Part of the body omitted. *Fl.*

Fig. 11. End of prophase. Five chromosomes plainly visible. *Fl.*

Fig. 12. Beginning of anaphase; two chromosomes are dividing transversely. Part of body omitted. *Fl.*

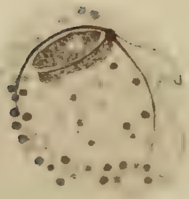
Fig. 13. Slightly later stage than the last. All the chromosomes are splitting. Note traces of the fibrillae. *Fl.*

Fig. 14. Approximately the same stage as Fig. 12, more deeply stained. At one pole are the short axostyle, one flagellum, and the root of another; at the other pole are the short, faintly indicated basal fibre and its membrane, in deeper focus than the plainer, longer, parabasal fibre. Cf. Fig. 10. *Fl.*

Fig. 15. Beginning of telophase. Traces of the chromosomes still visible. At one pole are the new membrane and basal fibre above and the old axostyle below the nucleus; at the other pole are the new axostyle above and the old parabasal fibre, still comparatively long, and the old membrane and basal fibre below the nucleus. *Fl.*

Fig. 16. Slightly later stage than the last, with nucleus resuming the prophase condition. Cytoplasmic structures probably as in the last, but insufficiently stained. Note blepharoplastic granules. *Fl.*

Fig. 17. Late telophase (?), deeply stained. At one pole, the old parabasal fibre, twisted partly out of focus, basal fibre and membrane below the nucleus, and above it the short, new axostyle; at the other pole, the old axostyle, regaining its length and staining affinities, new basal fibre and membrane and the short, new parabasal fibre. At both poles, the parabasal body is just evident. *Fl*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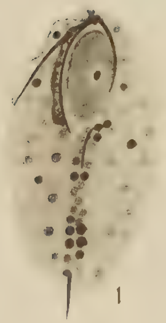
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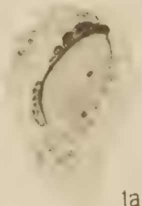
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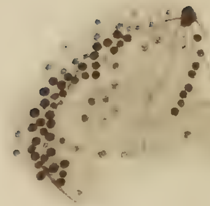
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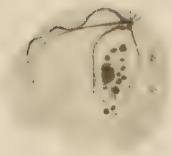
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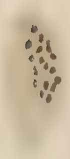
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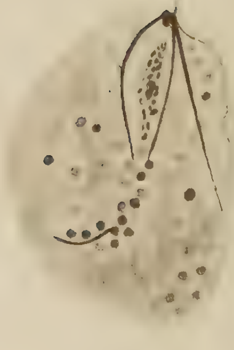
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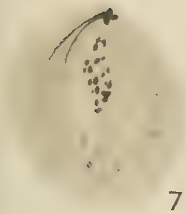
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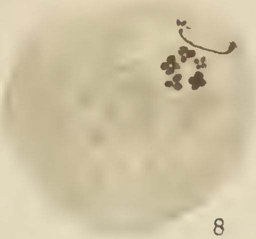
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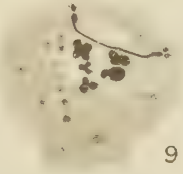
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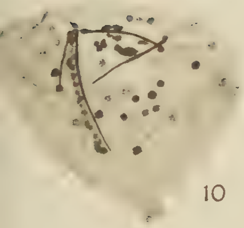
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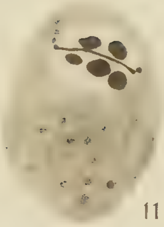
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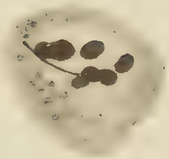
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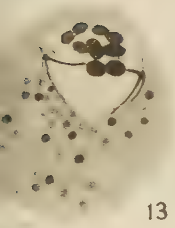
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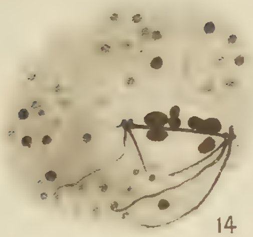
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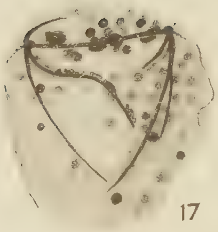
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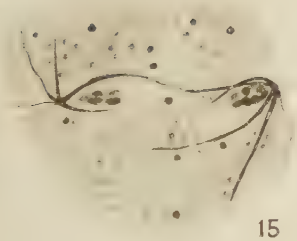
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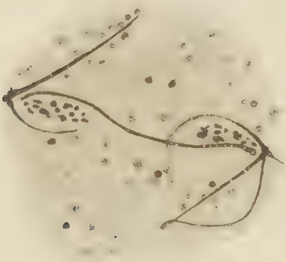
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I

Amoeba kerrii (n. sp.): Morphology, Cytology, and Life-History

BY

MONICA TAYLOR, S.N.D., D.Sc.

(From Notre Dame Laboratory and Zoology Department, University of Glasgow)

With one Plate and five Text-figures

INTRODUCTION

IN June 1939 a collection of plants from the fresh-water pools on the shore, in the vicinity of the Marine Biological Station, Keppel, was brought to ne by Miss Maureen McAlister. Since there were no crustaceans in the vater it was stored as a possible source of protozoa (Taylor, 1920). The lecaying material produced no crustaceans, a good omen. A few boiled wheat rains were added as a pabulum to supply the needs of the infusors which y this time were numerous, and Glasgow tap-water was used to compensate or evaporation of the original liquid. A glass plate covered the culture. In he summer of 1941 a more detailed examination of the material revealed he presence of amoebae easily visible under the low power of a Greenough inocular. The extreme opacity of these amoebae was arresting, the cause f it due to the fact that each was densely packed with cytoplasmic inclusions ncluding large numbers of crystals. The nucleus when in a position to be een stood out quite sharply, looking almost like a vacuole by contrast with he blackish cytoplasm. Further search revealed individuals not quite so lack in reflected light, probably because younger.

METHODS OF CULTIVATION

The original culture having been well stirred, several c.c. of it were put nto a Petri-dish (4 in. diameter) with 4 or 5 wheat grains and water. After months these cultures yielded no amoebae. One or two more trials also esulted in failure to establish Petri-dish cultures by this method. I next ried a technique which succeeds well in the case of *A. proteus Y*, i.e. of aking a large bulk of the original culture with 10 to 20 wheat grains and vater (bulk about 2 litres) and placing these in a cylindrical glass trough 3 in. in diameter by 4 in.). One only of these trials succeeded. However, he contents of the numerous failures were stored and did eventually produce few amoebae. The reason for the failure of the above technique will be bvious when the life-history is described, and the best method of securng spectacular cultures will be given below under the account of fission ivisions.

IDENTIFICATION AND CLASSIFICATION

In the light of much experience gained by a long study of large fresh-water amoebae I concluded that the dusky individual was new to science and that it belonged to Schaeffer's genus *Metachaos* (Schaeffer, 1926). I propose, however, to retain the name *Amoeba*, see below, for the genus and to give the specific name *kerrii*, in honour of my one-time teacher, now Sir John Graham Kerr, who long ago persistently urged the importance, for teaching purposes, of an investigation of the life-history and cultivation of the amoeba commonly called *A. proteus*.

My reasons for retaining the name *Amoeba* are:

1. Our scant knowledge of the life-history of free-living amoebae. More knowledge may modify a classification based on the study of the adult. For example, while *A. lescherae* and *A. proteus Y* would be placed in the genus *Chaos* because both possess longitudinal folds in the ectoplasm, their developmental forms are very different one from the other (see Taylor and Hayes, 1944, Text-fig. 5). On the other hand, the developmental forms of *A. discoides* and *A. kerrii* confirm the relationship based on a study of the adults.

2. The fact that the name *Amoeba* has become firmly embedded in the English language, and that Schaeffer's nomenclature has not yet been generally accepted even in U.S.A.

As will be shown later, the life-cycle of *A. kerrii* occupies 8 or 9 months. Apart from the developmental stages it presents quite remarkably differing appearances, these differences being due to age and physiological conditions. Full-grown individuals are those usually chosen by authors for descriptive purposes, but these vary. Therefore some standard for comparative purposes is essential. Taylor and Hayes (1944) suggest that the young adult should be chosen, this stage being defined as that in which fission division is regularly occurring. In no amoeba I have yet studied is the contrast between the mature and senile adult so great as in this. And since the longevity of the adult *A. kerrii* is considerable this aged adult may well preponderate in any 'catch' made during the summer and autumn in the open.

MORPHOLOGY OF MATURE TO SENILE ADULT STAGES

When transferred to a slide the amoeba of this stage reluctantly attaches itself and begins to creep by a few stout rounded, broad pseudopodia. The endoplasm is packed with crystals (Pl. 1); cuboid, square prisms, truncated bi-pyramidal in shape, which mask the presence of the large nutritive spheres, food vacuoles, and other cytoplasmic inclusions. Longitudinal folds of the ectoplasm are not present, hence the inclusion of *A. kerrii* in the 'Group' *Metachaos* (Schaeffer, 1926). The tip of a pseudopodium often bifurcates; the pseudopodia sometimes produce globular masses along the two sides. The ectoplasm is not voluminous, the contents of the endoplasm coming up close to the periphery. A cross-section of the middle region of a pseudopodium would be almost semicircular in outline.

The nucleus (Pl. 1) is always easily distinguishable in a creeping individual, being sometimes lenticular in shape but quickly turning over into a disk shape and then back again to the lenticular, as the creature progresses. The nuclear sap is very mobile. One gets the impression that the nucleus is large in comparison with the cytoplasm but it is difficult to measure the ratio (see below). Measurements of adult amoebae stained and mounted are 525 by 150μ, 375 by 105μ, 450 by 300μ; they are somewhat flattened out by the cover-slip.

In order to obtain some ratios between size of nucleus and cytoplasm, several amoebae of the same age were transferred to a slide and gently warmed. This had the effect of making each specimen more or less spherical. In this condition they were fixed, stained, and mounted. The ratio of the diameter of the nucleus to that of the spherical amoeba was so varied as to negative any comparative calculation of ratio. Moreover, as in *A. lescherae*, so in *A. kerrii*, the diameter of a spherical uninucleate differs little from that of a binucleate or even a 4-nucleated individual. Voegtlin and Chalkley (1930–45) obtained measurements by gently stimulating the amoeba by drawing and withdrawing it from a capillary tube until it had assumed a spherical form. They measured three dimensions of the nucleus and calculated for an ellipsoid. They concluded that volume of amoeba increases as the ratio of the cytoplasm to nucleus increases. But no corresponding measurements are available to give a comparative account of the cytoplasm nucleus ratio in all the large fresh-water amoebae.

When viewed *in situ* in the Petri-dish culture the majority of these stages, i.e. mature to senile, are floating.

THE YOUNG TO MATURE ADULT STAGES (Pl. 1)

These individuals have a greater surface area than obtains in the senile. They spread more rapidly and move more quickly when put on a slide under a cover-slip. The shape varies considerably. There is frequent reversal of direction in the flowing endoplasm of the various pseudopodia, an advancing pseudopodium often yielding its contents to another. The width of the advancing pseudopodium is often greater than that of the others. The young adult is much less dusky than the senile, crystals and nutritive spheres being smaller. Therefore at this stage *A. kerrii* bears a greater likeness to *A. proteus Y*, *A. lescherae*, *A. discoides*. There is often a little tuft of rounded, short, pseudopodia-like processes at the hind end which is reminiscent of the villi which give its name to *A. villosa*. In the latter amoeba, however, this structure is permanent. In *A. kerrii* this uroid is used as a pivot and appears in very young individuals a short time after they have hatched. The ectoplasm is more voluminous than in old individuals; a slight web can sometimes be recognized between adjacent pseudopodia. Two distinct regions can be recognized in the endoplasm of a rapidly moving amoeba under a cover-slip: a centrally placed, more densely packed, more active one, which gives the impression of a deep ravine down the middle of the pseudopodium, and a flanking region where the contents are more sparse and the movement slower.

This moving stream of endoplasm will flow on either side of an obstructing nucleus if the amoeba is well spread out (Hayes, 1938). The width of this active endoplasm varies in different individuals.

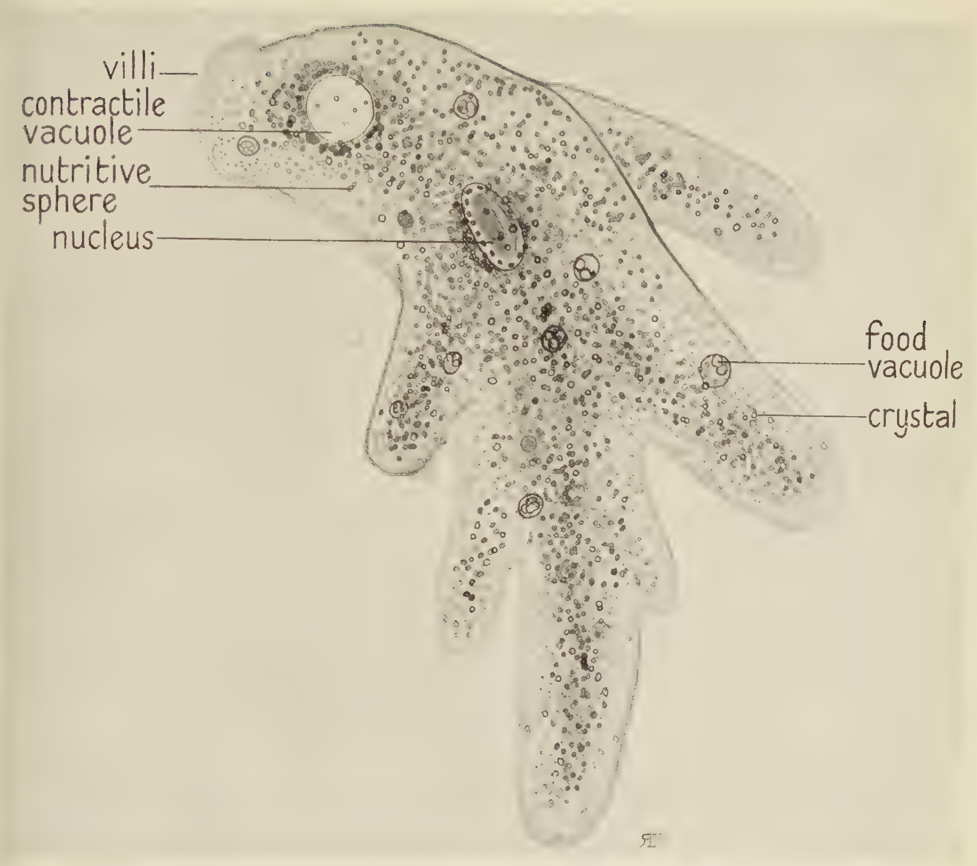
When given more freedom (e.g. studied in a live-box) with the help of a binocular eyepiece the pseudopodia of *A. kerrii* are seen to be arranged in definite tiers more or less parallel to the long axis. The contents of an advancing pseudopodium may be reversed or it may flow into a pseudopodium in a higher plane while a new pseudopodium is forming in a plane nearest the substratum. A criss-cross system of flowing endoplasm is the apparent result. But although the progressing and reversing streams of endoplasm are being continually developed, the amoeba as a whole disappears from the field of view. Sometimes the endoplasm ceases to flow and becomes piled up in one area giving the amoeba a lumpy appearance.

When studied *in situ* in Petri-dish culture with the help of a binocular eyepiece it can be observed that comparatively little of the amoeba is fastened to the substratum, a fact revealed by the passage of micro-organisms under the amoeba. The uroid, and peripheral portions of some pseudopodia, anchor the creature, while other pseudopodia emerge in all planes. Here again reversal of flow as well as progression are observable, and as before the amoeba goes out of the field of view. Feeding and moving phases alternate. Most of the food vacuoles are formed on the under side but are discernible from the upper side. The amoeba remains stationary while feeding sometimes for an hour or so. When the captured food organisms are dead the amoeba begins to move about actively again.

A Contractile Vacuole (Pl. 1) lies behind the nucleus. It evacuates very deliberately, and often, before this event, the newly forming vacuole is well on its way to attaining its maximum size. In one stained specimen the respective diameters of the old and newly forming vacuoles were 36μ and 28μ. Other measurements of the diameter of the contractile vacuole are 42μ, 44μ, 29μ, 39μ, the size depending on the age of the amoeba.

A. kerrii, by contrast with the other large amoebae, is not a voracious feeder under normal conditions. I have observed one specimen which lived in a damp chamber for 3½ weeks without food. The food organisms are ciliates, flagellates, rotifers. As in other related amoebae it possesses nutritive spheres which are small in young amoebae, larger in older ones, and can attain a diameter of 6·5μ.

The cytoplasm has a closer texture than that of *A. proteus Y* or *A. lescherae* and stains more deeply. It is much less vacuolated than the latter and much less easily ruptured than the former. This is especially well seen when a preparation containing several well-spread amoebae is irrigated with methyl green or aceto-carmine. When the fixative penetrates slowly great bladder-like distensions of the ectoplasm and a corresponding compression of the endoplasm occur. In some cases 'lines of force', very similar to those seen in the cytoplasm when a nucleus is dividing, are to be seen in this distended ectoplasm which rarely ruptures.



Freehand drawing of Amoeba kerrii (young adult)

The nucleus changes its form very readily, as previously stated. It has the same general plan of structure as that of the other species of amoebae (Taylor, 1930; Hayes, 1938) and will be described more fully later. It can be dissected away from the cytoplasm when its membrane has reached its maximum thickness.

When a 2-month-old Petri-dish culture is scrutinized under the lower powers of a Greenough binocular most of the individuals are of a more or less radiate type, some floating, some creeping. But there are also to be seen fission-spheres (see later) and a few outsized spherical individuals. These are multinucleate and are most numerous in an ageing culture. These outsized spherical multinucleate forms seem to be characteristic of all classes of amoebae—Clifford Dobell records them in parasitic amoebae. Their significance is obscure (see Taylor and Hayes, 1944, and Levy, 1924 and 1928).

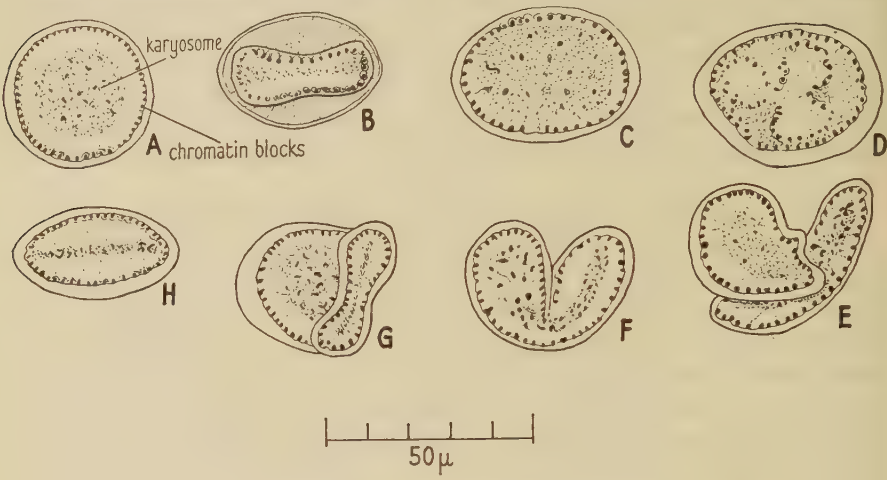
ACETIC ALCOHOL AND ACETO-CARMINE STUDIES

Aceto-carmine alone, and aceto-carmine after fixation in acetic alcohol, are valuable and quick reagents for the study of amoebae. At the outset these were employed. It was found that the cytoplasm of *A. kerrii* stains very deeply, a fact which militates against the usefulness of aceto-carmine as a nuclear stain. Fortunately the crystals are dissolved out by these reagents. Thus the remaining cytoplasmic contents can be more easily studied. In the light of one's former experience of amoeba nuclei, the most unlooked for result of aceto-carmine studies was the varying degrees of prominence exhibited by the nuclei of *A. kerrii.* Generally such an outstanding object in other amoebae, the nucleus of *A. kerrii* could sometimes be distinguished with difficulty from the surrounding cytoplasm. This was not always the case, however, especially when the nuclear membrane was stout. Lest these varying appearances were due to chemicals dissolved out of the numerous crystals, Bouin was employed as a fixative and the specimens were well washed in alcohol, which readily dissolves the crystals, and stained in Ehrlich's haematoxylin. The results confirmed the aceto-carmine investigation, some nuclei being outstanding and possessing thick nuclear membranes, in yet others the nuclear membrane was so thin as to be hardly discernible and the contents of the nucleus not sharply differentiated from the cytoplasm.

Aceto-carmine after acetic-alcohol fixation is useful for revealing the nutritive spheres. These lie in a vacuole, have a deep red rim, and a pale slate-coloured interior. The size of the nutritive spheres depends upon the age of the amoeba. An excellent method of demonstrating the abundance and size of the nutritive spheres is to fix and stain the amoeba with methyl green in acetic acid. The nutritive spheres stain green (see Taylor, 1939).

NUCLEUS OF A. KERRII (Text-fig. 1)

The nucleus in the main resembles that of the other large fresh-water amoebae. Its typical form is best seen in the young adult (A). Under the nuclear membrane are regularly arranged blocks containing chromatin separated by a clear space from a centrally placed disk-shaped karyosome, nuclear sap filling the interstices. The whole is extremely mobile, alternately oval or circular (cf. A and H) in outline as the amoeba creeps, with all manner of intervening shapes as the nucleus changes over from oval to circular 'view'. The karyosome (H) is band-shaped in the former (H) and disk-shaped in the latter (A). A scrutiny of large numbers of amoeba nuclei fixed in Bouin and stained in Ehrlich's haematoxylin reveals many interesting diversities other than that of outline. The most striking, as already mentioned, is the varied reaction to stain, and the varying thickness of the nuclear membrane. As already mentioned, when the latter is thick the nucleus can be removed entire from the cytoplasm. In ageing amoebae the nucleus tends to become deformed (D, E, F), the outline corrugated. 8-shaped figures are common and all appearances which so strongly suggest amitotic division are to be found.

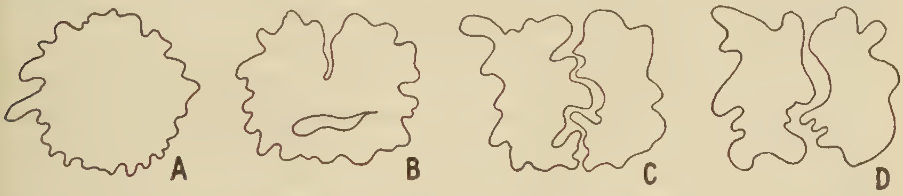


TEXT-FIG. 1. Nucleus of *Amoeba kerrii*

A. Typical nucleus of a very young adult. Surface view. B. Nucleus changing into 'side-view' position. C. Karyosome close up to chromatin blocks. D. Karyosome much vacuolated. E. Nucleus from an old individual, suggestive of amitotic division. F. A common appearance of the nucleus of an old adult. G. Nucleus rolling into 'surface-view' position. H. Nucleus in 'side-view' position.

FISSION DIVISIONS IN A. KERRII (Text-fig. 2)

In July 1943 a number of square, solid watch-glasses was assembled and into each was placed an adult amoeba taken at random from the parent culture along with a quantity of the culture fluid and food organisms. In every case the amoeba failed to divide. It however lived on for a varying period of time. These negative results were puzzling, especially as I had detected binucleate specimens in the stained preparations. Next I prepared cultures of the flagellates and ciliates found in the parent stock and upon which the amoebae were feeding and procured very young adults for the inoculation. Each amoeba was carefully washed and introduced into the watch-glass with a few food organisms and some fresh water. The next day most of the watch-glasses possessed two amoebae. Fission division obviously occurs only in the young adults. Once initiated, repeated fission produced a little colony of amoebae in each watch-glass. When the number approached about sixty-four I removed them to a Petri-dish (4 in. in diameter by ½ in.) supplying fresh water and food organisms and so obtained spectacular cultures. It was then found possible to add boiled wheat grains as a pabulum and so obviate the necessity of cultivating the food organisms separately. By subculturing I increased the number of my cultures, since access to almost unlimited numbers is necessary for the complete elucidation of the life-history.



TEXT-FIG. 2. Fission of Cytoplasm

A. Fission-sphere. B. Division has extended to equator of sphere. C. Division complete. D. Two daughter amoebae separate.

Fission of the Cytoplasm (Text-fig. 2) can easily be witnessed *in situ* or on a slide by removing one of the rounded morula-like individuals (= fission-spheres) from the culture (A). These fission-spheres adhere to the substratum while dividing in contrast to what obtains in *A. lescherae*. The break between the two daughter amoebae never stretches uninterruptedly from pole to pole (B) like a meridian, but lesser breaks along the meridian can be detected as the pseudopodia of each daughter amoeba form, and extricate themselves from each other (C). In one observation the whole process lasted half an hour. The two daughter amoebae remain very near each other for a time, again in contrast to *A. lescherae* where the two daughter components are very soon indistinguishable from the other members of the culture. At no time is there a long strand of protoplasm connecting the two as is so often figured in text-books.

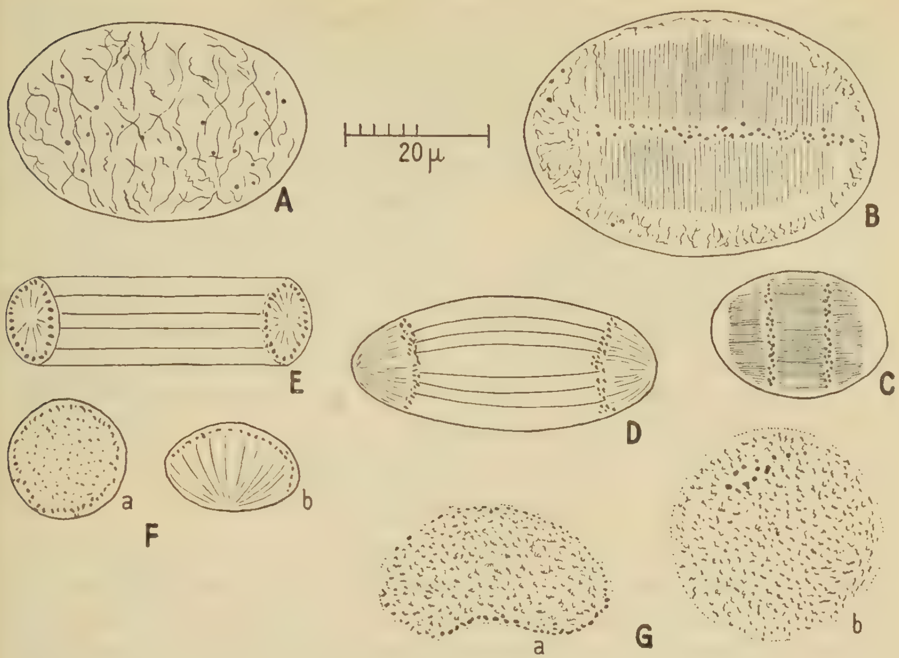
MITOSIS

The first record of mitosis in the large free-living amoebae was made by Sister Bernardine Carter (Carter, 1913). Since then it has been demonstrated in *A. proteus* (Dawson, &c., 1937), *A. discoides* (Taylor and Hayes, 1942), *A. lescherae* (Taylor and Hayes, 1944), *A. dubia* (Dawson, &c., 1935).

Method of obtaining Material to demonstrate Mitosis

Secure at least a dozen rich Petri-dish cultures where the amoebae are multiplying rapidly. Keep them as cold as possible until two or three hours before they are required, then raise the temperature to 70° or 80° F. Dividing amoebae will be present in sufficiently large numbers to warrant the making of permanent preparations. Remove each fission-sphere with a fine pipette to a slide. After providing each slide with three or four individuals in a minimum amount of liquid, gently lower a cover-slip provided with beeswax feet. The amoebae will grip the substratum. Irrigate quickly with Bouin's fluid, replace this by 90 per cent. alcohol, giving a good soaking in this and changing the alcohol in order to dissolve the crystals. After 70 per cent. alcohol stain in Ehrlich's haematoxylin, differentiate in acid alcohol, dehydrate in cellosolve, clear in xylene, and mount in Canada balsam. Examples of mitosis, if in a favourable position in the fission-sphere, stand out quite clearly under a No. 7 objective. Many of the mounted spheres show no nucleus; in yet others the mitotic nucleus is only discovered after much searching. In the former case the nucleus is deeply buried or masked by an overlying food vacuole. In any case the early prophase stages are difficult to see. The nuclear membrane is thin, and since the chromatin in an amoeba nucleus is very sparse, the early prophase is very often almost invisible (Text-fig. 3 A). The nucleus, of course, may be so placed on the slide as to be seen from the side, or foreshortened, but in a full-face view the outline is like that of a barrel. All the achromatic material gradually becomes arranged on a series of meridional lines, each composed of thick and thin lengths of material. Later (Text-fig. 3 B) these become little corkscrew-shaped masses as the chromosomes condense and make their way to the equator. Out of these corkscrew masses the definitive spindle-fibres are differentiated. These develop from the equator (B) and gradually spread to the poles. There is, of course, a great variety in the size of the dividing nuclei as there is in the resting ones, the size depending on the age of the amoeba and its volume, the larger the amoeba the larger the nucleus.

When the chromosomes are fully condensed at metaphase they lie on the equator in a clear area filled with a fluid-like substance that stains a bright pink in Ehrlich's haematoxylin. This surrounding pink area can be detected right up to late telophase. The chromosomes are very small and numerous. In anaphase (C and D) the barrel-shaped nucleus becomes more elongated. A few of the spindle-fibres, like the ribs of a barrel, stand out very conspicuously (D). The chromosomes still lying in the 'pink' area already referred to never reach the roof of the dome-shaped poles of the spindle (D and E) in telophase. In surface view, therefore, the chromosomes lie on the periphery of a circle just inside the nuclear membrane (F) in each daughter nucleus. At first the spindle-fibres can be distinguished (F, *b*), but these are gradually converted into corkscrew masses of achromatic material (F, *a*, G, *a*). The daughter nuclei are well on their way to reconstruction when the fission of the cytoplasm is completed (G, *a* and *b*). The nucleus absorbs liquid and so becomes larger. Around the periphery (G, *a*) the chromosomes can still be distinguished from the achromatic material. They become less distinguishable as the karyosome reforms. The nuclear membrane is thin and the whole



TEXT-FIG. 3. Mitosis in *Amoeba kerrii*

N.B.—The figures have been drawn from fission specimens of varying size.

A. A very early prophase. Achromatic structures lose their staining capacity, become arranged meridianwise: the sparse amount of chromatin makes the nucleus difficult to detect. B. From a large amoeba at later prophase. Chromosomes condensing out at equator, spindle-fibres becoming differentiated from the equator towards periphery.—Distinction between spindle-fibres and undifferentiated achromatic elements very pronounced. C. Early anaphase. Chromosomes lie in a clear area which stains a bright pink in Ehrlich's haematoxylin. D. Early telophase.—Some spindle fibres stand out very clearly.—The polar caps seen in side-view—spindle-fibres clearly marked. The area around the chromosomes stains bright pink. E. Telophase.—Polar caps seen in end view. Spindle-fibres more palely stained. Chromosomes around periphery still conspicuous. F. Daughter nuclei in an undivided amoeba. In *a*, end view of polar cap, the spindle-fibres have reverted back to achromatic fibres. In *b* they can still be detected. The coloured, clear region around the chromosomes makes them still a conspicuous object. G (*a* and *b*). From an amoeba fixed as soon as the fission of the cytoplasm was completed and examined in aceto-carmine. Nuclear membrane so thin as to be almost invisible. Chromosomes, in their pink-coloured area becoming less distinctly differentiated—the rest of the nucleus a clearly stained, homogeneous mass of discrete elements.

difficultly distinguishable from the surrounding cytoplasm. (N.B. This want of prominence of the nucleus must not be confused with that described previously.)

It is interesting to note that in a large number of the rounded off, large, multinucleate amoebae the nuclei are often near the periphery and very close to each other. They have all the appearances of newly constructed nuclei. What causes the failure of the cytoplasm to divide after the nucleus has divided remains still to be explained.

An observation made on a fission division may throw light on the phenomenon that occurs in all the amoebae I have studied, namely the decrease in its size as the amoeba becomes senile. In old cultures pieces of non-nucleated amoeba-cytoplasm occur. Yet I have never observed any large-scale occurrence of the casting off of lumps of cytoplasm by amoebae which might account for the presence of these non-nucleated fragments. However, on one occasion I had transferred a large fission-sphere of *A. kerrii* to a slide to study fission. The amoeba gave every sign of dividing into two larger and equal protoplasmic masses, and one smaller. The two larger and equal-sized portions were true daughter amoebae—the smaller portion was a non-nucleated mass. This may have been due to the mechanical disturbance involved, though it is not likely, as scores of fission-spheres so transferred have proceeded to divide normally; but it might also explain how ageing amoebae can become progressively smaller.

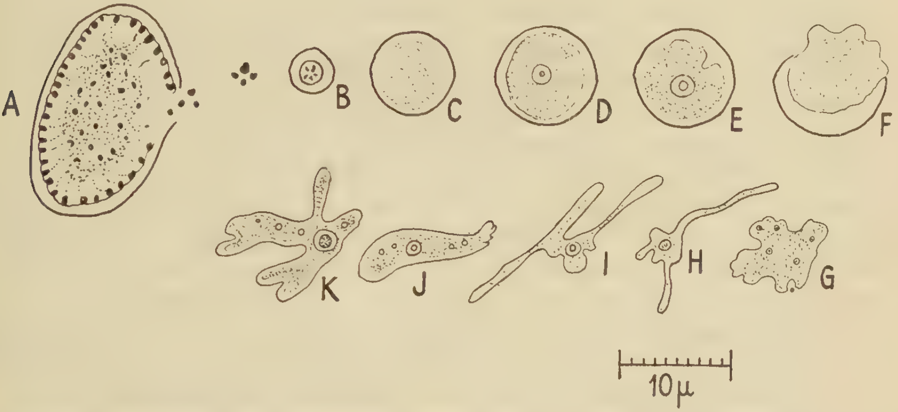
REPRODUCTIVE CYCLE

In his brilliant and comprehensive work on parasitic amoebae, Dobell (1928, 1938) has found no trace of a sexual cycle. No such cycle has been found in *A. proteus* (= dubia) (Carter, 1915), *A. proteus Y* (Taylor, 1924), *A. discoides* (Hayes, 1938), *A. lescherae* (Taylor and Hayes, 1944), and in the amoeba now being described, i.e. *A. kerrii*. On the advice of Dr. Helen Pixell Goodrich, to whom I have been indebted in the past for much constructive criticism, the clumsy and ugly-sounding nomenclature used in the description of *A. lescherae* (Taylor and Hayes, 1944) and in *A. discoides* (Hayes, 1938) has been abandoned, because of this absence of a sexual cycle. The mature amoeba which produces ripe cysts is referred to simply as a mature amoeba and the newly hatched individuals as amoebulae. (See also *A. discoides* (Quart. J. micr. Sci. **87**, 195), where the nomenclature has been emended.)

The *Fission Cycle* in the life-history of *A. kerrii* is succeeded by that of the *Reproductive Cycle* when each mature amoeba becomes converted into a number of encysted young. The process starts with the emission of chromidia (i.e. small masses of chromatin material on an achromatic base) from the nucleus into the cytoplasm (Text-fig. 4 A) where they become the rudiments of the encysted young. The rudiment grows, as can be seen by studying progressively more mature amoebae. In the meantime the nutritive spheres in those amoebae which are preparing to form encysted young become less numerous, their substance becoming absorbed into the general cytoplasm. Under the influence of the nucleus rudiment cytoplasm is formed around it and eventually a cyst-wall encloses this small nucleated mass, i.e. the encysted young (Text-fig. 4 B and C). Hundreds of these cysts are formed from every ripe adult. Eventually the cytoplasm of the latter disintegrates and the cysts are liberated into the surrounding water where they remain for a period of time.

Method of Procuring Microscopical Preparations in the Reproductive Cycle

Select old well-conditioned individuals from a successful four months' culture, fix in acetic alcohol and then stain in aceto-carmine. Or, fix in aceto-carmine and give several washings of the fixative to dissolve out the crystals. Chromidia and all stages in the formation of encysting amoebae can thus be secured.



TEXT-FIG. 4. Cyst Formation.—Hatching of young *A. kerrii.*—Early stages of Development

A. An adult nucleus.—Nuclear membrane absorbed where chromidia are escaping into cytoplasm of a mature amoeba. B. and C. Differentiation of encysted young amoeba. D. Cyst nearing the time of hatching, nucleus visible. E. Encysted amoeba with a functioning contractile vacuole. F. Escaping amoebula, nucleus not easily visible. G. Newly hatched amoebula feeding—several food vacuoles. H. Ectoplasm has become more fluid. Long anchor-like pseudopodia enable the creature to grip the surrounding debris. I. Older individual. J. Limax-like creeping amoebula. K. Floating amoebula.

EXCYSTATION OF A. KERRII

Method of Procuring Cysts for Excystation

A pure line culture of *A. kerrii* was set up at the end of July 1943 by placing one young adult in a solid watch-glass with culture fluid and water containing food organisms grown separately. When the progeny numbered about sixty-four a Petri-dish was prepared with culture fluid and food organisms for its reception. Food organisms were supplied regularly as required. At the end of August two freshly boiled wheat grains were introduced. The culture was spectacular at the end of October when fresh wheat was added, the old grains being removed to make a subculture, as they were carrying a number of adhering amoebae.

In January 1944 the culture was undergoing a period of depression. Cysts were abundant. These were studied *in situ* by means of a No. 7 water-immersion lens or on a slide. It is a matter of experience to decide which cysts are ready to excyst. I have had specimens under observation for two weeks before hatching took place. A fully differentiated cyst measures 9 μ, though there is a slight variation in size. The wall is stout and in contrast to the other large amoebae that have been described, it is single (Text-fig. 4 C, D). When the time of hatching approaches the nucleus is easily visible, centrally placed and surrounded by endoplasm which gradually becomes more and more granular. The periphery of the encysted amoeba does not look like an inner cyst-wall; hence, as explained above, the cyst-wall of *A. kerrii* is single.

Shortly before the actual excysting takes place, a contractile vacuole begins to function (Text-fig. 4 E). The amoebula must therefore be imbibing water and the cyst-wall must be ruptured. After some time the contents of the cyst become obscured by a bluish-tinged oily-looking substance, the ectoplasm. Gradually this latter may be seen oozing out of the cyst, which slowly becomes empty as a more or less limax-shaped amoeba escapes. This moves for a distance of about 45 μ and commences feeding on very small bacteria present in the water (G). It is extremely well camouflaged as it lies thus for a varying period of time. The empty cyst-wall remains circular in outline for a long time after the escape of the amoebula, a proof of its toughness. However, when the excystation takes place in the direction of the cover-slip the irregular circular outline of the breach can be detected. It measures about 3 μ.

In a day or two the excysted amoebulae develop a more fluid ectoplasm (H) and long slender pseudopodia are extruded. These hook on to debris, which fact suggests that they are a device for transporting the amoebulae to new pastures (H and I). However, when placed on a slide these floating forms may be made to creep (J). When viewed *in situ* the floating forms with elongate pseudopodia are seen to change over to the creeping type (J) or round up. The protrusion of blunt pseudopodia first from one side and then from the other as the amoebula progresses, is somewhat explosive and rapid in character.

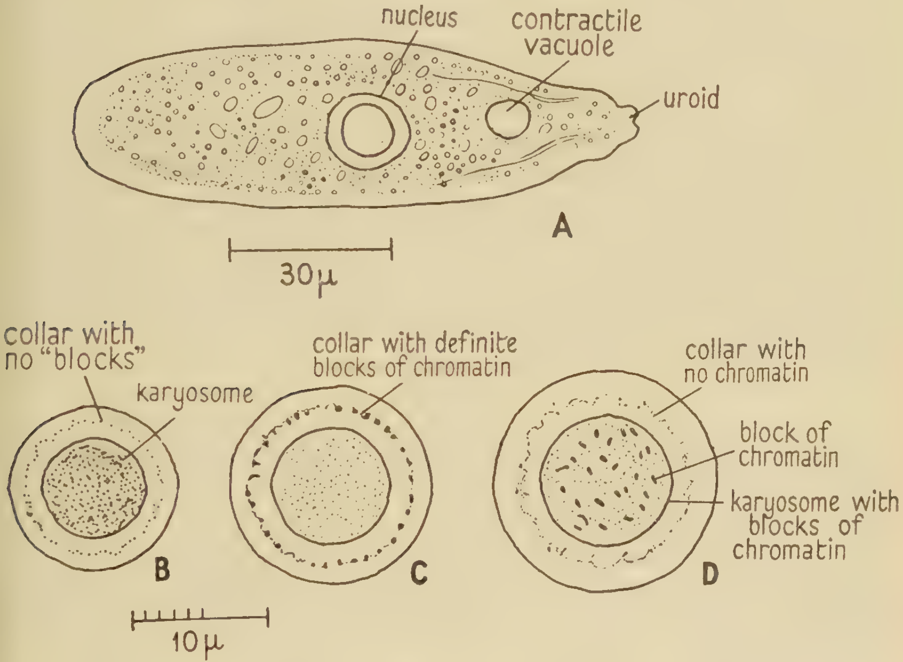
FURTHER DEVELOPMENT OF A. KERRII (Text-fig. 5 A)

The amoebula grows slowly, the endoplasm becoming provided with inclusions of various kinds. The limax shape of a creeping form of 100 μ is superficially similar to that of *A. proteus Y* save that there is no 'sole', i.e. no precursor of longitudinal folds. In it nucleus and contractile vacuole and a small uroid are easily distinguishable. These developing young are much stronger than those of *A. proteus Y*. As in the adult, so in the developing stages, floating and creeping phases alternate.

NUCLEUS OF DEVELOPING A. KERRII (Text-fig. 5)

A karyosome surrounded by a clear area of nuclear sap enclosed in a nuclear membrane is the structure of the nucleus as seen in the living amoeba. Further details are demonstrated by staining methods. The ratio of the diameter of the karyosome to that of the nucleus is greater than in that of *A. proteus Y*, *A. discoides*, and *A. lescherae*. In preparations made with aceto-carmine or permanent preparations stained in Ehrlich's haematoxylin the consistency of the karyosome varies from a distinctly granular to a smooth more or less homogeneous one.

Lying in the clear space between karyosome and nuclear membrane, at a varying distance from the latter, is a collar of achromatic material which eventually becomes apposed to the nuclear membrane. On this collar (Text-fig. 5 B, C, D) are sometimes to be seen distinct granules of chromatin. When this 'collar' has reached the nuclear membrane the developing young amoeba may be considered to have attained the adult stage. The possession of the 'collar' marks off the immature amoebae of *A. proteus Y*, *A. discoides*, *A. lescherae*, *A. kerrii* from the Mayorellas.



TEXT-FIG. 5.

A. A young amoeba in creeping position. B, C, D. Nuclei of developing *A. kerrii*.

To Mr. Ronald W. Graham Kerr who responded so graciously to my request that he should undertake the filial task of executing the text-figures and plate, I offer my warm thanks and appreciation of his skill.

DIAGNOSIS

Found in ponds. Size increases with age up to a maximum length in locomotion of 576μ. It then decreases as the amoeba becomes senile. The pseudopodia are blunt; few, in senile specimens; the ectoplasm has no longitudinal folds, hence resembles that of *A. discoides* and Schaeffer's genus *Metachaos*. The endoplasm is densely packed with crystals and nutritive spheres giving a more dusky appearance than obtains in the other fresh-water amoebae yet described. Contractile vacuole about 40μ in a young adult. Food: flagellates, rotifers, ciliates. Floating and creeping forms occur, the former often radiate or spherical except in senile individuals when they are amoeboid. When feeding the amoeba creeps. The species resembles *A. proteus Y*, *A. discoides*, *A lescherae* in the possession of a discoid nucleus presenting a round or band-shaped outline as it is rolled about by the endoplasm. Nuclear division mostly resembles that of *A. discoides*, and in the main, the prophase, metaphase, and anaphase of *A. proteus Y*, but the dome-shaped telophasic elements are in contrast to the cone-shaped ones of *A. lescherae*. Fission divisions are restricted to the young adult and do not occur in ageing individuals.

The species differs from *A. proteus Y*, *A. dubia*, *A. discoides*, *A. lescherae* in its longevity [senile adults linger on in the ponds until winter], in the marked difference between the appearance of a young adult and a senile individual; in the dusky character which is progressively intensified, as old age approaches, by the growth in size of the nutritive spheres and crystals; and by the diminished size of the senile. Another difference is the greater ease with which the reproductive cycle can be traced in *A. kerrii*.

SUMMARY

1. An amoeba characteristically blackish when viewed under the low power of a Greenough binocular in reflected light was discovered and isolated from macerating water weeds found in fresh-water pools on the shore near the Marine Biological Station of Keppel.

2. A study of its morphology revealed its relationship to Schaeffer's genus *Metachaos*. For reasons given above, the name *Amoeba* has been retained for the genus and the specific name *kerrii* has been given in honour of Sir John Graham Kerr.

3. The life-history has been worked out by means of pure-line cultures.

4. The cyst measures about 9μ in diameter. The newly hatched amoebulae grow slowly and develop eventually into young adults which, because of the small size of their crystals and nutritive spheres, are much less dusky than the mature individuals first discovered. An average adult size in creeping is 504 by 576μ.

5. The nucleus, which divides by mitosis, has the same general build as that of *A. discoides*, *A. proteus Y*, *A. lescherae*. Immediately under the nuclear membrane is an achromatic network containing chromatin blocks and connected with a more or less centrally placed karyosome, the whole immersed in nuclear sap. The nucleus assumes varying shapes as it is rolled about by the streaming endoplasm.

6. Morula-like spheres (= the fission-spheres) give rise to two daughter amoebae by cleavage along a meridional central plane. Fission is confined to young adults.

7. A fission cycle is succeeded by a reproductive cycle. Any mature amoeba can give rise to encysted young which are formed by the action of

chromidia escaping from the nucleus. The cyst-wall is single. Hundreds of encysted amoebulae may be formed from one mature amoeba. No sexual stages occur.

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The Histochemical Recognition of Certain Guanidine Derivatives

BY

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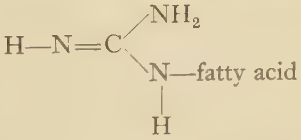
INTRODUCTION

SAKAGUCHI'S test for arginine depends on the production of a pink or red colour when certain guanidine derivatives are treated with α-naphthol and sodium hypochlorite in alkaline solution (Sakaguchi, 1925). This test had been familiar to biochemists for 19 years before it was independently adapted to histochemical use in the same year by Serra (1944) and myself (1944). Our results were published on opposite sides of the battle-front. The purpose of the present paper is to discuss the validity of Sakaguchi's test and to present my histochemical adaptation of it in its latest form.

THE VALIDITY OF SAKAGUCHI'S TEST

Sakaguchi's test was applied to a number of substances by himself and by Poller (1926). The chemical composition of the red substances formed in the test is uncertain. Sakaguchi gives an analysis of the reaction-product with glycocyamine, but Poller questions the correctness of his formula.

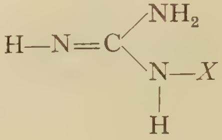
Sakaguchi thought that his test gave a positive reaction with all substances having this structure:



but not with any substance in which the hydrogen atoms shown in the formula are replaced by any other atoms or groups of atoms.

Poller showed that the hydrogen of the H—N= group, and *one only* of the hydrogens of the NH_2 group might be substituted by methyl, without interfering with the positive reaction. He also showed that a number of substances containing no fatty acid radicle give a positive reaction.

Weber (1930), who did not test any substance not previously tested by Sakaguchi or Poller, drew the tentative conclusion that a positive reaction is given by all substances having the formula:



where X is either a fatty acid or an alkyl radicle. This, however, does not cover all known cases. For instance, the hydrogen of the H—N= group may be substituted by methyl, as in trimethylguanidine, yet a positive reaction is given. Further, a positive reaction is given by dicyandiamide, in which X is neither a fatty acid nor an alkyl group.

A study of all the accurately named substances investigated by Sakaguchi and Poller shows that a generalization can be made as to the chemical formula of positively reacting substances, more exact than those made by either of these authors or by Weber. The following substances react positively:

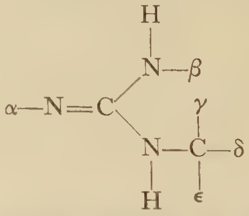
Dicyandiamide, monomethyl-, dimethyl-, and trimethyl-guanidine, glycocyamine, alacreatine, α-guanidino-*n*-butyric acid, galegine, agmatine, and arginine.

Negative results are given by:

Guanidine, asymmetrical dimethyl- and trimethyl-guanidine, creatine, creatinine, alacreatinine, glycocyamidine, nitroguanidine, nitroarginine, hydantoin, methyl hydantoin, hydantoic acid, thiohydantoic acid, biuret, urea, uric acid, allantoin, alloxan, guanine, cyanuric acid, histidine, tryptophane, asparagine, tyrosine, lysine, and ornithine. (Poller also obtained negative results with a number of unnamed nitrogenous compounds, including various other mono- and diamino-acids, but the absence of particulars prevents this information from being used in forming a generalization.)

The facts given above are covered by the following generalization:

A positive result with Sakaguchi's test is given by all substances, so far as is known, having the following formula:



where α is H or CH_3 and β is also H or CH_3. A positive result is not given, so far as is known, by any substance in which this is not so.

The three valences represented by the lines connecting a carbon atom with γ, δ, and ϵ may be satisfied by various atoms or groups of atoms, or all three may be taken up by a single nitrogen atom, in dicyandiamide.

In the present state of knowledge, a positive reaction with anything occurring in any plant or animal tissue must be regarded as indicating the presence of arginine (free or combined), galegine, or agmatine. If further research shows that an appreciable amount of any of the other positively-reacting substances is present in any particular tissue, this conclusion must be modified accordingly. Arginine is, of course, one of the most widely distributed substances in the tissues of organisms, since it occurs in most proteins. Galegine and agmatine, on the contrary, are extremely restricted in distribution. The former was discovered by Tanret (1914) in the seeds of *Galega officinalis* (Leguminosae) and has not been found in other plants. Agmatine was discovered by Kossel (1910) in the testis of the herring. It was subsequently found by Engeland and Kutscher (1910) in ergot. It has also been reported by Kiesel (1924) in ears of rye that are free from ergot. In the present state of knowledge it is reasonable to regard a positive reaction with the test as indicating the presence of arginine (free or combined), except in studies of *Galega officinalis*, ergot, ears of Gramineae, and the testes of the herring and allied fishes.

THE ADAPTATION OF SAKAGUCHI'S TEST TO HISTOCHEMICAL USE

General Remarks

The test succeeds best with paraffin sections. The alkali, however, tends to loosen the sections from the slide, and it is therefore best to attach them with a film of celloidin.

In Sakaguchi's original test the α-naphthol was used at a very low concentration, because it tends to become yellowish in the absence of arginine, and this discoloration is evident when the solution is viewed in considerable thickness in a test-tube. In the test described below, only a thin section is examined, and free α-naphthol is not present in appreciable amount when the observation of colour is made. There is therefore no advantage in using an extremely dilute solution.

The amount of sodium hypochlorite used was determined from the following considerations. If much is used, colour develops very rapidly, but the excess of hypochlorite soon destroys the colour. The object was therefore to find the minimum necessary to produce the full colour. Since ordinary hypochlorite solutions are very unstable, the proprietary product 'Milton' was used. This contains sodium hypochlorite at approximately 1 per cent., together with sodium chloride and small quantities of other salts. Milton is remarkably stable, and the salts other than sodium hypochlorite do not affect the test. Where Milton is not available, a strong solution of sodium hypochlorite should be diluted until it gives full coloration in approximately fifteen minutes, when substituted for Milton in the test described below.

For the quantitative determination of arginine it is desirable to have very rapid development of colour, and for this reason Weber (1930) substituted sodium hypobromite for hypochlorite. The rapid coloration given by hypobromite is followed by rapid fading, and Weber used urea to eliminate the excess of hypobromite. In adapting Sakaguchi's test to histochemical use, Serra (1944, 1946) followed Weber in using hypobromite and urea. I preferred not to do so, for three reasons. (1) The validity of the test rests upon Sakaguchi's and Poller's studies, which were made with hypochlorite. (2) Hypochlorite is much more readily available than hypobromite. (3) Rapid development of colour is not necessary in non-quantitative studies.

The amount of α-naphthol solution and Milton used in the test is measured by drops. There is no point in using more accurate measures, for the exact amounts of α-naphthol and hypochlorite necessary to achieve the full reaction and leave none over depend on the amount of arginine (or other positively-reacting substance) in the section to be tested; and this will obviously vary widely in different cases.

The concentration of sodium hydroxide is kept fairly low, so as not to damage tissues.

In this test I introduce the use of pyridine as a mounting medium. It gives good definition not only in this test, but with basic dyes and dye-lakes in general. The refractive index at the D line is 1·509. Pyridine may be used alone, or chloroform (refractive index 1·449) may be mixed with it. The purpose of the chloroform is to prevent the solution of the film of celloidin that holds the section in place.

Detailed Description of the Histochemical Test

Almost any routine fixative may be used. Zenker's and Bouin's fluids, Heidenhain's 'Susa', mercuric-acetic, and formaldehyde-saline are all suitable. Zenker's fluid seems slightly preferable to the others. The following solutions are also required:

Celloidin, 1 per cent. in a mixture of absolute alcohol and ether in equal volumes.

Sodium hydroxide, 1 per cent. aqueous.

α-naphthol, 1 per cent. in 70 per cent. alcohol.

Milton (or another sodium hypochlorite solution of the same strength).

A mixture of 3 volumes of pyridine with 1 volume of chloroform.

The test is performed as follows:

I. Place a piece of tissue (preferably not more than 3 mm. thick) in one of the fixatives mentioned above. Leave for 24 hours.

II. Wash out, dehydrate, &c., as usual, and embed in soft paraffin wax.

III. Cut sections at 16μ, and attach them to slides with Mayer's glycerine and albumen.

IV. Remove the wax with xylene and wash in absolute alcohol.

V. Dip the slide in the celloidin solution. Wipe the back of it. Drain the slide and let the alcohol and ether evaporate partly (but not completely).

VI. Pass the slide through 90 per cent. and 70 per cent. alcohol to water.

If the fixative contained mercuric chloride, give the usual treatment with iodine-alcohol and sodium thiosulphate at this stage.

VII. Put 2 c.c. of the sodium hydroxide solution in a watch-glass. Add 2 drops of the α-naphthol solution, and then 4 drops of Milton. Mix the fluids by drawing them into and pressing them out of a pipette several times.

VIII. *Without waiting*, remove the slide from water, jerk the water off it (the front of the slide cannot be wiped, as a cloth would destroy the celloidin film), lay it flat, and flood it with about 1 c.c. of the mixture made at stage VII. Leave the slide for 15 minutes. The colour develops gradually and reaches its maximum in this time.

IX. Drain the fluid off the slide. Blot the slide with filter-paper and place it in a jar of the pyridine-chloroform mixture. Leave for about 3 minutes.

X. Mount in pyridine-chloroform (or in pyridine). If it is wished to keep the slide for more than an hour, it is best to paint round the edges of the coverslip with gold size.

Result. A pink or red colour indicates the presence of arginine (free or combined) or some other positively-reacting guanidine derivative.

The colour is not permanent. Full intensity is retained for several hours and the colour then fades gradually. Very strongly reacting objects still show colour after the lapse of some weeks.

Instead of flooding the slide with the mixture at stage VIII, one may advantageously use Gurr's staining plate.

Results of the Application of the Test to Tissues

Since most proteins contain arginine, protoplasm gives a positive reaction. Most of the so-called tests for proteins are so weak when applied to sections that ordinary ground-cytoplasm is often uncoloured or only slightly tinged. It is generally not very easy to demonstrate by histochemical methods one of the most fundamental facts of biology—that cytoplasm contains protein. The Sakaguchi test, as modified for histochemical use, generally gives clear evidence of the existence of arginine in ground-cytoplasm. In some cases (e.g. in the epithelial cells of the mammalian intestine, or the spermatocytes and young spermatids of the frog) the reaction is quite strong. Smooth muscle also reacts fairly strongly. The reaction is naturally much stronger in the aleurone grains of plants, on account of the higher concentration of protein. The aleurone grains in the cotyledons of the apple-seed (Fig. 1 A) give a particularly strong reaction and provide a suitable object for a first trial of the method by botanists. (The testa of the seed must, of course, be removed before fixation, and the cotyledons should be cut across to allow the fixative to enter easily.) Chromatin is usually more darkly stained than nuclear sap and cytoplasm, and nuclei therefore stand out slightly in most tissues. The nuclei of the spermatozoa of many animals react more strongly than any other cell-constituent that I have studied. I have already mentioned those of the snail in my earlier paper (1944). Those of the newt (Fig. 1 B) and salamander give a particularly powerful reaction. The testis of the newt is the most suitable object for a first trial of the method by zoologists. The organ may be taken at any time between late summer, when spermatogenesis is complete, and the beginning of the breeding season in spring. The powerful reaction in the nuclei of spermatozoa accords with the high arginine-content of the basic proteins that occur there in combination with nucleic acid.

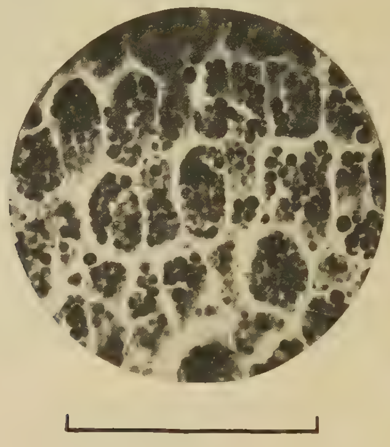


FIG. 1 A.

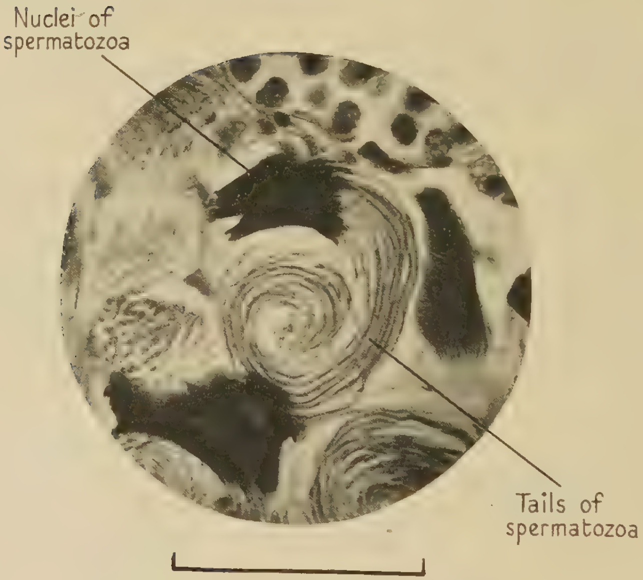


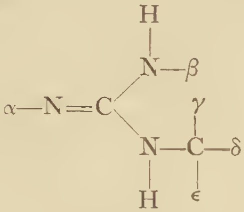
FIG. 1 B.

FIG. 1. Photomicrographs of sections stained by the modification of Sakaguchi's test described in this paper. The scale drawn below each photomicrograph represents 0·1 mm.
A. Part of the cotyledon of an apple-seed.
B. Part of the testis of a common newt (*Triturus vulgaris*).

The staff of the Botanical Garden at Oxford kindly provided me with some seeds of *Galega officinalis*. These showed nothing remarkable when subjected to the test. No method is known for the histochemical distinction between galegine and arginine. Klein and Schlögl's method (1930) for the microchemical identification of galegine has not been adapted to histochemical use. The cells of the cotyledons showed large, strongly reacting aleurone grains, which may have contained galegine as well as combined arginine. (It is stated by Muller (1925) that the seeds of *Galega officinalis* contain no free arginine.)

SUMMARY

1. A review of known facts indicates that Sakaguchi's test gives a positive reaction with all substances having this formula (and with no others):



where α is H or CH_3 and β is also H or CH_3.

2. Arginine, being a constituent of most proteins, is by far the commonest positively-reacting substance that occurs in the tissues of plants and animals. Galegine and agmatine, which also react positively, are of very restricted occurrence.

3. Details are given of a method whereby Sakaguchi's test may be applied to paraffin sections.

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Ester Wax: A New Embedding Medium

BY

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1. INTRODUCTION

THE aim of this investigation was to produce new ribboning embedding media which would, as far as possible, combine some of the advantages of paraffin wax and celloidin with a reduction of their disadvantages.

The research required the selection and manufacture of many organic products. Thanks are expressed to Dr. T. Malkin, Dr. C. E. H. Bawn, and Dr. G. T. Young of Bristol University and to Dr. E. Cockbain of the Imperial Chemical Industries for help and advice on the preparation of uncommon esters.

The research was conducted under the supervision of Professor C. M. Yonge.

2. HISTORY OF RIBBONING EMBEDDING MEDIA

Paraffin wax as a ribboning embedding medium has been known since 1882. It has been the main embedding medium ever since, but has been subject to additions and modifications at various times. Diethylene glycol distearate as a ribboning medium was first investigated by Orton and Post (1932) and later by Cutler (1935), but they abandoned the material because it had a number of weaknesses.

3. DEFINITION OF TERMS

Before proceeding to deal with the difficulties which arose in the course of this research, definitions of some of the terms employed in the technique of sectioning are necessary.

Section. This is a thin slice of material embedded in a suitable matrix. It is a single slice, cut on a microtome and may vary in thickness from 1 to 60μ.

Section compression. Taking paraffin wax as an example, if the edge of the block at right angles to the knife is measured, and the corresponding edge of a section cut from that block is measured, it will be found that the length of the section is smaller than the side of the block. This diminution is due to compression of the embedding material as it passes the knife edge. It may amount to as much as 33 per cent. of the original breadth of the block, increasing inversely with the thickness of the section. It appears to be due to the crystalline nature of the embedding matrix, the particles of which slide upon one another with ease as compared with the particles which go to make up an amorphous material such as celloidin.

Section de-compression. This means the expansion of previously compressed material towards its original length. In actual measurement it is rare for de-compression to equal compression, so that even when sections have been carefully treated on hot water there are still some traces of compression left.

Section expansion. This means an increase in either the length or breadth of a section after flattening on warm water as compared with the corresponding side of the block. It is almost unknown.

Section crumpling. This is a puckering of the section in any direction or plane.

Section curling. The section curls upon itself, a condition which may be carried to a point at which a cigar-like cylinder is produced.

Section corrugation. This is a form of crumpling in which the puckering takes place in an even series of lines parallel with the knife edge.

Section breakage. Very thick sections may break into a number of jagged bars roughly parallel with the knife edge.

Section flattening. This means the reduction of a crumpled, curled, or corrugated section to flatness, commonly by floating the section on warm water. Such flattening is usually accompanied by an increase in length, an increase which must not be confused with the increase due to de-compression. Only those sections which are flat as they leave the knife can be used for studying compression or de-compression.

Ribbon. A ribbon is a series of consecutive sections firmly joined to one another. The sections comprising it should be flat, easy to enumerate, and the ribbon easy to handle. Thus any embedding material from which ribboning is expected must show both cohesion and adhesion. There may be doubts as to the number of adhering sections which properly constitute a ribbon. It has been decided in this paper that a minimum of ten sections is a reasonable figure.

Section range (*Range of thickness of single sections*). This is an expression of the performance of a wax of stated composition or melting-point at a given room temperature. The range varies with the room temperature and is also influenced by the sharpness of the knife. It refers to single sections only.

Ribbon range (*Range of thickness of section-ribbons*). This indicates the ribboning performance of a wax of stated composition or melting-point at a stated room temperature. It is generally a smaller range than the section range of the same wax, because the factors responsible for successful ribboning are not the same as those required for successful production of single sections. It is dependent on a wide variety of factors which include room temperature, block temperature, age of the block, knife sharpness, interference by static electricity, &c.

4. Production of Ester Wax

In the production of ester wax, paraffin wax was taken as type of embedding matrix and after consideration of the technical problems involved the following list was compiled of the essential properties of any new ribboning embedding medium. The material should be:

1. Other than a hydrocarbon
2. Soluble in alcohol, dioxan, &c.
3. Suitable for sectioning and ribboning
4. Molten between 30° C. and 50° C.
5. Translucent or transparent
6. Stable
7. Homogeneous
8. Harmless to embedded material
9. Capable of being flattened after ribboning
10. Non-poisonous
11. Easy to handle
12. Cheap
13. Odourless
14. Colourless.

No natural wax was found which combined all these properties. Pure, refined waxes or fats were less suitable than mixed esters. Finally five synthetic materials were selected as possible bases:

Diethylene glycol distearate
Diethylene glycol monostearate
Glyceryl tristearate
Glyceryl monostearate
Stearin.

Of these substances diethylene glycol distearate proved to be the most satisfactory. It was used as the main constituent of a series of mixtures which were made to combat its crystallinity and to prevent air from creeping in during the making of blocks. Over 200 reagents were used, and after more than 2,000 mixtures had been tested the following was found to be satisfactory:

Diethylene glycol distearate	73 gm.
Ethyl cellulose, low viscosity	4 gm.
Stearic acid	5 gm.
Castor oil	8 gm.
Diethylene glycol monostearate	10 gm.

As a softening agent castor oil was inferior to octadecanediol diacetate, but as the latter had to be made specially it was finally abandoned.

The following are among the main physical characters of this mixture:

Melting-point	48° C.
Section range	2–20 μ at room temperature of 66° F.
Ribbon range	2–15 μ at room temperature of 66° F.
Compression after flattening	7·6 per cent. at 10 μ.

The above mixture may be varied according to the tissue to be embedded. For hard tissues the ethyl cellulose content may be increased to 5 per cent; for very hard material up to 7 per cent., but above 7 per cent. the mixture becomes too viscous to be used. The higher the amount of ethyl cellulose present the longer is the infiltration required.

If a soft wax is required, as much as 20–30 per cent. of diethylene glycol monostearate may be used.

A description of the constituents is given below:

Diethylene glycol distearate. This is a commercial product which contains a high proportion of diethylene glycol dipalmitate. It frequently contains dust and gritty impurities which may be removed by filtering through a hot filter funnel using a rapid filter paper. A good paper is Barcham Green 904.

It is better to filter the main ingredient in this way rather than the final mixture because the viscosity of the ethyl cellulose makes filtering a slow process. Exposed to the air the ester develops a bloom, which is composed of crystals, but which does not appear to affect the inner parts. This bloom also makes its appearance on the surface of the embedding mixture.

Ethyl cellulose. This is a white, very light, powdery substance, which may be obtained in three grades of viscosity—low, medium, and high. There is little advantage in increasing the viscosity of any embedding medium and the low viscosity grade was used. A certain amount of dust is present in the commercial product, and this can be removed only by allowing it to settle in the mixture and decanting off the clear liquid. The presence of ethyl cellulose in the mixture imparts toughness to the section, enables the ribbon to be handled easily, and prevents the sections melting or coming to pieces on the water when flattening.

Stearic acid. This is the purified form of stearin, and was used in all the experiments. It assists the mixture by promoting greater homogeneity, and by improving its ribboning properties.

Castor oil. The purified, colourless product is preferred. It is the softening agent in the mixture.

Diethylene glycol monostearate. This is a soft, lard-like ester, which improves the texture of the mixture, and assists ribboning. It generally contains free diethylene glycol which may be removed by melting and decantation.

To make the mixture for embedding, first weigh out the castor oil into a porcelain pot with a handle. Then weigh out the diethylene glycol distearate and add about 15 gm. of the 73 to the castor oil. Melt over a bunsen flame and when hot add the ethyl cellulose, already weighed out. Heat until this dissolves and then add the rest of the diethylene glycol, the stearic acid, and diethylene glycol monostearate. Allow to cool and the mixture is ready.

On being made into blocks it forms a translucent wax-like substance. If the pure wax used for making the block is heated approximately 10°–15° C. above its melting-point and then poured into the mould a clearer block will be produced than one in which the wax was used when just at its melting-point. The specimen should be in another container and should be put into the block when it has cooled sufficiently for a skin to form next to the metal L-pieces.

5. Ester Wax Solvents

Ester wax is designed so that the use of hardening hydrocarbons, either for clearing or embedding, may be avoided. A representative selection of clearing agents or solvents follows. The figures indicate the minimum temperature at which ester wax is soluble in all proportions with the given fluids.

Table 1. *Temperatures at which Ester wax is soluble in pure solvents, and in aqueous dilutions*

I Wax insoluble; IM Solvent immiscible with water; S Wax soluble at all temperatures; Results in degrees Centigrade.

Solvent	*Percentage of water*				
	0	*10*	*20*	*30*	*40*
Alcohols					
Methyl	I	..	..	..	..
Ethyl	43	I	..	..	..
iso-Propyl	38	45	50	I	..
n-Butyl	38	IM	..	..	..
Amyl	36	IM	..	..	..
Octyl	33	IM	..	..	..
Methylated spirits	43	I	..	..	..
Ethylene glycol	I	..	..	..	..
Propylene glycol	I	..	..	..	..
Butylene glycol	I	..	..	..	..
Terpineol	22	IM	..	..	..
Ethers					
Ethyl ether	S	IM	..	..	..
Ethylene glycol mono-ethyl ether	38	65	I	..	..
Ethylene glycol mono-methyl ether	65	I	..	..	..
Ethylene glycol mono-butyl ether	34	40	44	45	I
Diethylene glycol	I	..	..	..	..
Diethylene glycol mono-ethyl ether	I	..	..	..	..
Diethylene glycol mono-butyl ether	38	45	48	I	..
Dioxan	S	46	I	..	..
Esters					
Methyl acetate	34	IM	..	..	..
Ethyl acetate	S	IM	..	..	..
iso-Propyl acetate	S	IM	..	..	..
n-Butyl acetate	S	IM	..	..	..
Amyl acetate	S	IM	..	..	..
Ethylene glycol diacetate	I	..	..	..	..

TABLE 1 (contd.)

Solvent	*Percentage of water*				
	0	*10*	*20*	*30*	*40*
Esters (cont.)					
Glyceryl mono-, di-, and tri-acetates	I	..	..	..	..
Ethyl butyrate	S	IM	..	..	..
Ethyl lactate	40	75	I	..	..
Butyl lactate	33	IM	..	..	..
Amyl lactate	32	IM	..	..	..
Benzyl benzoate	33	IM	..	..	..
Ethylene glycol mono-ethyl ether mono-acetate	33	IM	..	..	..
Hydrocarbons					
Xylene	S	IM	..	..	..
Benzene	S	IM	..	..	..
Toluene	S	IM	..	..	..
Ligroin	S	IM	..	..	..
Chlorinated hydrocarbons					
Chloroform	S	IM	..	..	..
Carbon tetrachloride	S	IM	..	..	..
Pentachlorethane	S	IM	..	..	..
Ethylene dichloride	S	IM	..	..	..
Ethylene chlorhydrin	S	IM	..	..	..
Ketones					
Acetone	33	I	..	..	..
Diacetone alcohol	40	I	..	..	..
Cyclohexanone	20	I	..	..	..
Amines					
Anilin oil	18	I	..	..	..
Aldehydes					
Cinnamaldehyde	33	IM	..	..	..
Anisaldehyde	33	IM	..	..	..
Salicylaldehyde	25	IM	..	..	..
Natural oils					
Arachis	S	IM	..	..	..
Castor	S	IM	..	..	..
Cedarwood	S	IM	..	..	..
Clove	S	IM	..	..	..
Olive	S	IM	..	..	..
Origanum	S	IM	..	..	..
Peppermint	S	IM	..	..	..
Wintergreen	S	IM	..	..	..

From the figures given in Table 1 it is clear that ester wax is soluble in a wide range of substances, any of which could be used as clearing agents. The following are suggested:

Ethylene glycol mono-ethyl ether (Cellosolve).
Ethylene glycol mono-butyl ether.
Diethylene glycol mono-butyl ether.
Dioxan.
Cedarwood oil.

6. INFILTRATION WITH ESTER WAX

As with paraffin wax specimens may be taken directly from the wax solvent into the molten wax. This is not recommended. The more gradual change effected by interposing a solution of ester wax in the solvent before finally placing in molten wax is preferable.

The correct time during which a specimen should remain in molten ester wax depends on the size of the specimen, but a few representative examples are given below.

Frog egg, 2·5 mm. diameter . .	3 hours
Pieces of *Lumbricus*, 15 × 15 mm. (diam.)	15 hours
Pyloric stomach, Cat, 15 × 15 × 15 mm. .	24 hours
Slice of brain, Cat, 34 × 25 × 10 mm. .	60 hours

In all cases it is advisable to change the specimen from one bath of molten wax to a second and a third before finally making the block. This is particularly important when using clearing agents of low evaporation rates, e.g. dioxan.

The presence of free stearic acid in the mixture leads to the production of copper stearate should copper pans be used. This does not appear to affect the tissue, but a piece of paper placed at the bottom of the copper pot containing the molten wax will prevent contact with the copper stearate. Porcelain pots are recommended, however.

The times taken for producing stained serial sections of *Hydra oligactis* may be of interest.

Fixed Carnoy . .	5 min.	Staining and flattening .	5 min.
Washed, dehydrated, and cleared in 'Cellosolve' .	3 „	Drying	10 „
Infiltrated, 2 baths . .	5 „	Wax removal, counterstaining and mounting . .	7 „
Block-making and cooling	10 „		
Ribboning, 6μ . .	15 „	Total	one hour.

7. BLOCK-MAKING WITH ESTER WAX

The well-marked crystalline nature of diethylene glycol distearate makes block-making with ester wax a careful operation. Air infiltration is the chief difficulty. The following procedure, using metal L-pieces, was found most useful.

Adjust the L-pieces to the size of the block required on a small piece of glass such as a 3 × 1 in. slide. Pour the molten ester wax into them. Take the specimen from molten wax in the embedding oven and place it in the wax in the L-pieces so that it lies on the skin of congealed wax next to the glass slide, and in the angle of one of the L-pieces rather than in the centre. Place the slide with the L-pieces on it in a Petri-dish of cold water, so that the block is not submerged, and as cooling and contraction of the wax takes place fill in the hole which forms either by using a hot spatula or needle or by dropping molten wax into it. A slight amount of air infiltration will take place at the top of the block, but this is usually so far away from the specimen that it gives no trouble and is removed when the block is trimmed.

The quicker a block cools the better will be its texture. It is always preferable to have the L-pieces cold before pouring molten wax between them, because a skin of wax is quickly produced next to the cold metal. As contraction of the wax takes place it can shrink away from the sides of the mould, and in this way the centre of the block may contract less. A better block is produced if the wax used in making it is heated over a bunsen to about 20° C. above its melting-point just before pouring into the L-pieces.

8. CUTTING ESTER WAX SECTIONS

All the precautions to be taken when cutting sections of paraffin wax must be observed when cutting sections of ester wax. In addition sections of ester wax must be cut *very slowly*, otherwise badly crumpled sections will be produced.

It is in the cutting of ester wax sections that one of the fundamental differences from paraffin wax may be observed. Paraffin wax is made up of a number of minute crystals and as the block passes the knife edge the crystals can be felt grating upon it. In addition the movement of the crystals produces a shortening, i.e. compression, of the section. This compression, even though followed by de-compression when the section is placed on warm water, is certainly harmful to embedded tissues. The smooth appearance of celloidin sections is considered due not only to the avoidance of heat in the technique but also to the fact that celloidin is not crystalline and that compression is practically non-existent.

Ester wax closely resembles celloidin in this respect. Its very large crystals adhere to one another so firmly in the properly cooled condition that it behaves as an amorphous mass even though the crystals are visible under the microscope and are much larger than those of paraffin wax. The size of a slowly cooled crystal may be about 4 mm. in diameter; the commonest size is 2–3 mm.

A sharp microtome knife is essential, and it should be stout and securely held. Ester wax is a very hard material compared with paraffin wax and a microtome knife which will not bend away from a block of paraffin wax during cutting will frequently do so when ester wax is used. Even a trace of looseness in the bearings of sliding parts of the microtome will lead to sections of unequal thickness, and this will produce poor ribboning or no ribboning at all.

For thick sections the material may be cut on the microtome the day the block is made, but thin sections of 3–6 μ are better obtained on the second or third day.

For very thick sections and for block trimming it is an advantage to place the block in a warm oven or in warm water for a minute or two.

9. FLATTENING SECTIONS OF MATERIAL EMBEDDED IN ESTER WAX

It has already been stated that one of the best embedding mixtures is:

Diethylene glycol distearate	73 gm.
Ethyl cellulose, low viscosity	4 gm.
Stearic acid	5 gm.
Castor oil	8 gm.
Diethylene glycol monostearate	10 gm.

The object of this research was to produce a wax which was not a hydrocarbon and which had a greater water tolerance than paraffin wax. The above formula and the section on ester wax solvents shows that this has been done. But increased water tolerance in an embedding medium also implies a greater affinity for water by the sections, and also a differential affinity, because the tissue in the section is much nearer its natural condition than when embedded in a hydrophobic wax and during flattening on warm water takes up more water than the wax. This differential intake leads to crumpling of the section so that when it is finally dried after flattening on water small folds are present.

This was the case with material sectioned in the mixture given above. The sections, both wax and specimen, were flat when on warm water on a slide, but crumpled when the slide cooled later on. The possibility that successful flattening of the specimen might be induced by lowering the surface tension of the flattening water, by introducing substitutes for stearic acid in the formula, and by adding a water-soluble wax solvent to the flattening water was therefore investigated.

Reagents such as sulphonated castor oil and sodium taurocholate were used for lowering the surface tension. Stearic acid esters were substituted for stearic acid in the formula. Cellosolve and ethyl lactate were included in the water-soluble wax softeners. Variations in the pH of the flattening water were also tried.

The results showed that no beneficial effects followed from the use of such reagents, and further experiments were abandoned.

A noticeable feature of ester wax is the tendency for individual sections of a ribbon to separate during flattening. This frequent fault was considered as being due to a low margin of ribboning power. Such a margin could possibly be increased by further experiments with crystalline materials such as stearic acid, but even then the greater the amount of adhesive material added the more would the special properties of diethylene glycol distearate be reduced. Accordingly no further experiments were made in this direction.

Considerable improvement in flattening was noticed when the sections were placed in an oven at 35°–40° C. after flattening on water in the usual way. In this manner they dried at the temperature at which they were flattened and were not allowed to become cold and wrinkled. It is essential that ester wax sections should be dried in this manner, and that when placed in the oven there should be sufficient water beneath the ribbon to act as a lubricant.

10. STAINING SECTIONS OF MATERIAL EMBEDDED IN ESTER WAX

Because of its greater water tolerance it is possible to stain sections of material cut in ester wax in two ways:

A. In the ribbon, i.e. with the wax still present;
B. After removal of the wax, as with paraffin wax sections.

The second method is well known and needs no description. The steps of the first method are as follows:

1. Smear a microscope slide with albumen in the usual way.
2. Flood the slide with a solution of methylene blue in water (0·001 per cent. or stronger). Place the ribbons on the solution and flatten by warming.
3. Drain away the excess staining solution and then wash the slide with the section still on it by adding a few drops of water and draining again. Repeat this until no more blue comes away.
4. Leave sufficient water on the slide to float the ribbons and place in an oven at 45°–50° C. to complete the flattening and to dry. Twenty minutes to one hour is enough. Any of the nuclear coal-tar dyes may be used in this way.

For removing the wax and counterstaining the following solutions are necessary:

	1	2	3	4	5	6	7	8
Cellosolve (Ethylene glycol mono-ethyl ether)	..	..	10	20	40	80	100	100
Ethyl acetate	..	..	45	40	30	10	..	..
Xylene	100	100	45	40	30	30	..	..

Erythrosin or eosin may be used as a counterstain dissolved to saturation in any solution from No. 4 to No. 7. The higher the percentage of cellosolve present the more will the methylene blue be extracted. The ordinary procedure is outlined below:

1. Partly remove wax from the sections with xylene, 2–5 minutes.
2. Continue this process in the 10 per cent. cellosolve mixture.
3. Differentiate the methylene blue in mixture No. 6 or No. 7.
4. Counterstain in No. 8, to which has been added eosin or erythrosin to saturation. If it is found that the methylene blue is extracted, the lower percentage cellosolve solutions with eosin to saturation should be employed instead.
5. Transfer the slide from No. 8 to No. 4, bringing a good supply of stain on the slide. This stain will then be forced into the material owing to the decreased solvency of the No. 4 mixture. Rinse well to remove excess stain solution.
6. No. 3 solution for 2–4 minutes.
7. Xylene (two baths).
8. Mount.

11. MOUNTING STAINED ESTER WAX SECTIONS

After counterstaining, sections are placed in pure xylene and mounted in the usual way. Canada balsam in xylene is not recommended as methylene blue invariably fades in this medium. The proprietary product 'Sira' and the polystyrene mounting medium put forward by Kirkpatrick and Lendrum (1941) do not make methylene blue preparations fade. They are recommended in place of balsam in xylene.

Stained preparations should be kept out of direct sunlight or very bright daylight.

12. SUMMARY

Ester wax is a new ribboning embedding medium. It consists of five substances which may be varied in proportion so that media of different characteristics may be produced. A mixture which will meet most requirements is as follows:

Diethylene glycol distearate .	73 gm.
Ethyl cellulose	4 gm.
Stearic acid	5 gm.
Castor oil	8 gm.
Diethylene glycol monostearate .	10 gm.

The following facts relate to this mixture:

Melting-point. 46–8° C.

Section range. 2–20μ at a room temperature of 64° F.

Ribbon range. 2–15μ at a room temperature of 64° F.

Final compression loss. 7·6 per cent. at 10μ (after flattening).

Solvents. Alcohols, ethers, esters, ketones, hydrocarbons, aldehydes, chlorinated hydrocarbons, natural oils, &c.

Cutting. This must be done more slowly than with paraffin wax.

Flattening. Sections may be flattened on tap-water or aqueous staining solutions. Flattening must be completed in a drying oven at 40–5° C. with sufficient water under the sections to float them.

Staining. Two methods: (1) In the ribbon; (2) As for paraffin wax sections.

Pre-mounting fluid. Xylene, benzene, ligroin, &c.

Mounting fluid. 'Sira', or Kirkpatrick and Lendrum's polystyrene mounting medium.

Conclusion. It is believed that ester wax may open up new methods in histology and cytology. Its greater water tolerance and its property of ribbon staining followed by drying constitute an advantage found in no other embedding medium. Greater use of the wide range of solvents as stain carriers is indicated.

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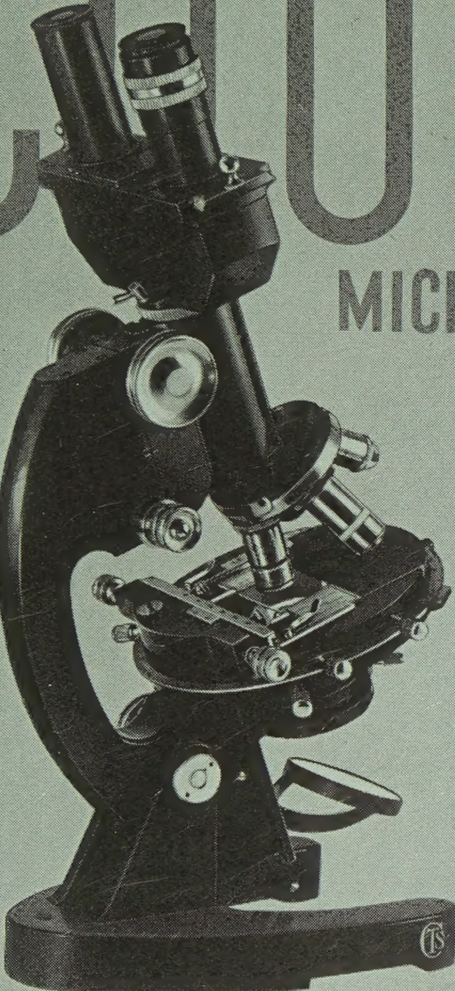

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